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CORRECTION.

On page 150, Vol. iii, No. 2, November 20, 1920, line 18, for *The resistance after, etc.*, read *The resistance due to A and M after, etc.*; line 20, of the same page, equation (4) should read as follows:

$$\text{Resistance} = 2{,}475.22\left(\frac{K_A}{K_M - K_A}\right)\left(e^{-K_A T} - e^{-K_M T}\right) + 70.69\left(e^{-K_M T}\right) + 10 \quad (4)$$

page 151, line 11, equation (5) should read as follows:

$$O = 89.1\left(\frac{K_N}{K_O - K_N}\right)\left(e^{-K_N T} - e^{-K_O T}\right) - 90\, e^{-K_O T} \quad (5)$$

line 16, of the same page, for *the value of O is 92.57* read *the value of O is 82.57*; lines 16 and 17, of the same page, for $(92.57 - 10) \div (100 - 10) = 0.917$ read $(82.57) \div (90) = 0.917$.

NUMERICAL LAW OF REGRESSION OF CERTAIN SECONDARY SEX CHARACTERS.

By A. PÉZARD.

(From the Laboratory of General Biology of the College of France, Paris.)

(Received for publication, October 22, 1920.)

Postpuberal Castration.

When a young cock is deprived of its genital glands (prepuberal castration), it is found that the fleshy appendices which the head bears (Fig. 1), *i.e.* the comb, barbules, and "oreillons," do not develop, or that at least they follow the general law of growth of the body; moreover, instead of acquiring the brilliant color which they have in the normal adult cock, they remain pale and are covered with a fine, white film due to epithelial desquamation (farinaceous appearance).[1] Since these organs attain complete development when minute fragments of testicle are inserted into the peritoneum of castrated fowl, or when frequent injections of fresh testicular extract are given, we conclude that they are related to an internal secretion produced by the male genital glands. The question arises whether, once the comb and similar organs have developed, the testicle is still necessary to assure their maintenance.

To determine this point we have castrated not only cockerels but also adult cocks in full possession of their secondary sex characters. Regression of the comb begins during the first days following operation (Fig. 2). It is at first rapid but gradually diminishes until at the end of a few weeks (5 to 12) the comb and similar organs reach a stable condition in which they present the reduced size and farinaceous aspect which characterize those of the castrated fowl of our first series. This result demonstrates conclusively that the internal secretion of

[1] Pézard, A., Le conditionnement physiologique des caractères sexuels secondaires chez les Oiseaux, *Bull. biol. France et Belg.*, 1918, lii, 1.

the testicle is just as necessary for the maintenance of these organs as it is for their growth.

In these experiments on postpuberal castration, the length and width of the comb were measured each week during the period of

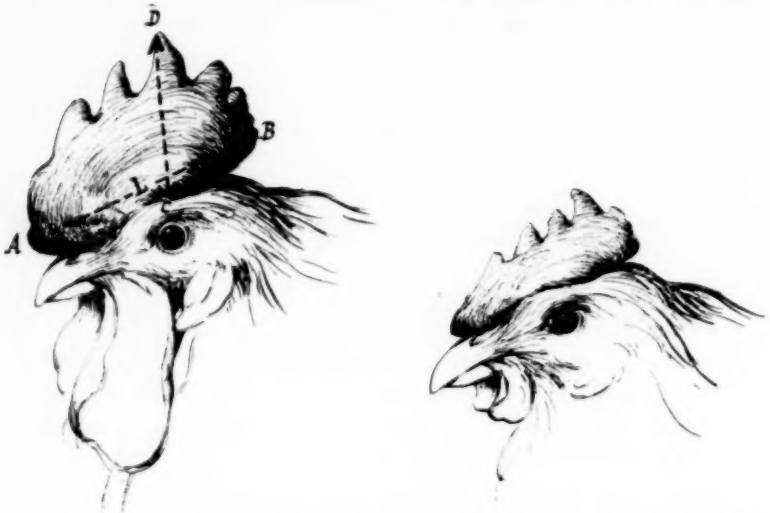

FIG. 1. Head of a normal adult cock (left) and head of a castrated cock (right).

FIG. 2. Successive aspects of the comb of a cock during regression: (1) at the time of castration; (2) 5 weeks later; (3) 7 weeks later; and (4) at the end of regression.

regression. The length (Fig. 1) was measured from the anterior extremity (A) to the most distant point of the posterior portion (B); the width from the posterior point of origin (C) to the edge of the highest point of the crest (D). In this way the measurements are

always relative to the same segments; the dimensions are given in millimeters. It was difficult to secure greater precision than this, nor would that inform us except on the secondary variations not susceptible of modifying the general appearance of the phenomenon. It should be noted, however, that the dimensions relating to length are relatively more precise than those having to do with width, since in the former case we were dealing with a larger figure.

A curve has been plotted representing the variation in length of the comb during regression. This curve may also represent the variation in volume, for the three dimensions were found to be reduced proportionately, as shown by careful observation of the organ in process of regression, which visibly retains the same form during the period of its etiolation. This observation may, moreover, be mathematically verified by measuring the linear dimensions and the weight of the comb (the latter being proportionate to the volume) in normal and in castrated males. The following is an example.

Nov., 1919. Normal cock. Comb, 73.40 mm. Weight, 8.8 gm.
Castrated " " 54.28 " " 3.1 "

If the proportions of the comb remained the same, the weight should be proportionate to the cubes of the homologous dimensions. This is found to be true.[2]

$$\left(\frac{73}{54}\right)^3 = 2.45 \quad \left(\frac{40}{28}\right)^3 = 2.91 \quad \frac{8.8}{3.1} = 2.84$$

We encountered a difficulty in determining the time of regression. Regression, at least when it follows postpuberal castration, occurs immediately after the operation (indicated on the figures as *Op*). On the other hand, the end of the regressive period is somewhat uncertain. In our experiments we have arbitrarily considered the regression as terminated when the comb undergoes oscillations of no more than a millimeter per week.

[2] This does not mean that the equation between the length of the comb and the time is exactly that between the volume and the time; nevertheless, it is easy to pass from one to the other.

Results.

In the following paragraphs we shall indicate in each case: (1) information relating to the bird under observation; (2) a graphic chart on which are shown the observed curve and the parabola which most nearly approximates it; the latter departs from the horizontal at the point where regression begins and becomes tangent to the horizontal at the point where regression ends. This parabola may be represented algebraically by the formula

$$L = l + \tfrac{1}{2}C(\theta - t)^2$$

in which L represents the length of the comb at a given time during regression, l the length of the comb at the end of regression, C an individual constant, θ the total period of regression, and t the time corresponding to the unknown ordinate L.

Experiment 1.—Cock, Dorking, about 1 year old. Sex characters well developed; combs and barbules scarlet; sexual instincts well developed.

Castrated Mar. 20, 1912. Both testicles very large, total weight 42 gm. Regression of the comb began at once and was finished at the end of 12 weeks (Fig. 3). There was coincidence between the observed curve and the parabola throughout almost the entire length of the curve: only at Point 6 of the ordinate was there a deviation, regression having been accelerated by a cause unknown to us; after that the acceleration was compensated for, since the phenomenon resumed its normal curve.

Experiment 2.—Cock, Beauceronne, about 6 months old. Secondary sex characters in course of development but not completely developed. Comb brilliant but not yet of its final length. Completely castrated Jan. 18, 1912. Regression began immediately and continued for 7 weeks (Fig. 4.) There was the best possible coincidence throughout the entire extent of the curve.

The weight curve (shown by the broken line, the scale of weight being found at the right) indicates clearly that the curves are independent of each other and that the phenomenon of regression follows its regular course irrespective of the general nutrition of the bird. Regnault and then Pflüger later have shown that the body regulates its consumption not according to the substances which are offered it but according to the demands of the cells. Here we see clearly that the needs of the cells are in their turn influenced by the testicles, and that their nutrition is independent of the general metabolism.

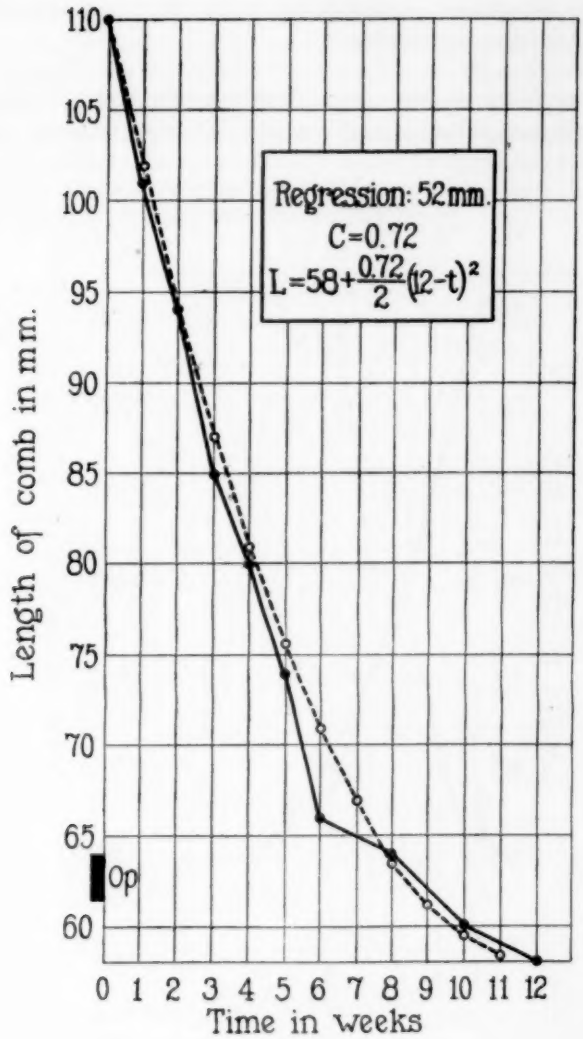

FIG. 3.

Experiment 3.—Cock, Black Orpington, about 8 months old. Castrated Dec. 4, 1909. The bird, whose sexual growth was slow, did not yet possess its secondary sex characters. It received from Jan. 17 to May 23, 1910, frequently repeated injections of testicular extract of the undescended testicle of a pig. Under this influence the comb developed and reached its normal length (86 mm.).

On May 23 the injections were discontinued, and on May 26 the comb began its regression, which ended in July (Fig. 5).

Coincidence is particularly remarkable in the present case. Note that the last injection had been given May 23; regression did not actu-

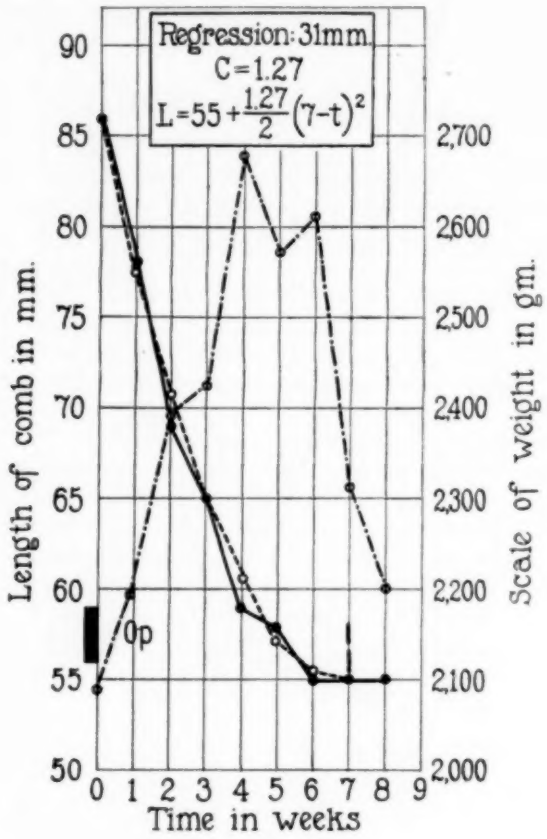

FIG. 4.

ally begin until May 26. This retardation is explained, in our opinion, by the slowness of absorption of the product introduced into the body intraperitoneally, owing possibly to the colloidal nature of this product.

Experiment 4.—Cock, Faverolle, hatched July 1, 1913, began to display the secondary sex characters in Nov., notwithstanding an attempt at castration later recognized as incomplete. Two testicular nodules about the size of a hazelnut were extirpated Feb. 26, 1914; regression began immediately; we shall consider it as ending at 6 weeks (Fig. 6). Coincidence was not so good as in the other cases, for reasons which we are not in a position to explain at present; it should be added that during the 3 months following (May to July) regression continued but was negligible, being only 3 mm.

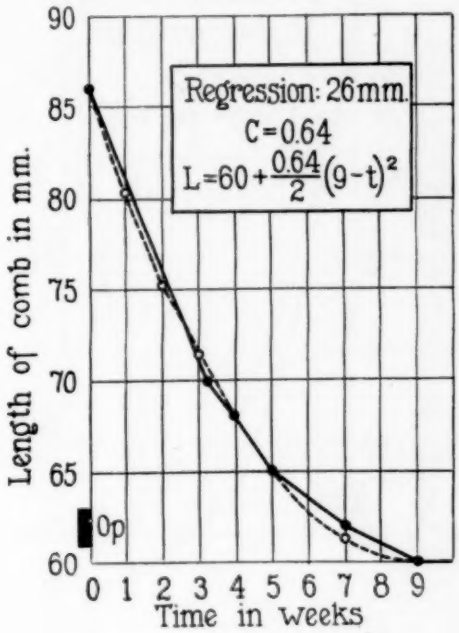

FIG. 5.

Experiment 5.—Cock, Bare-neck, hatched July, 1912. Castrated Nov. 11, 1912. Received in right peritoneal cavity fragments of ovary from a pullet of the same brood. These fragments did not appear at once to have any influence on the regression of the comb, which was effected at the normal rate. On the other hand, a small testicular nodule developed after the operation, and from the 7th week the phenomenon was reversed and a new growth of the comb brought about, hence the second branch of the curve. At autopsy this nodule was extirpated: the weight was 1.3 gm. It had the usual testicular structure. Although the two segments of the curve do not appear to correspond, the regression was complete (Fig. 7).

The coincidence between the recorded and the theoretical curves is good in the lower points, and less satisfactory at the time corresponding to Points 2 and 3, when there was an acceleration of regression. The portion of the curve AB corresponds to the regression, the segment BC to the renewed development of the comb under the influence of the nodule of regeneration.

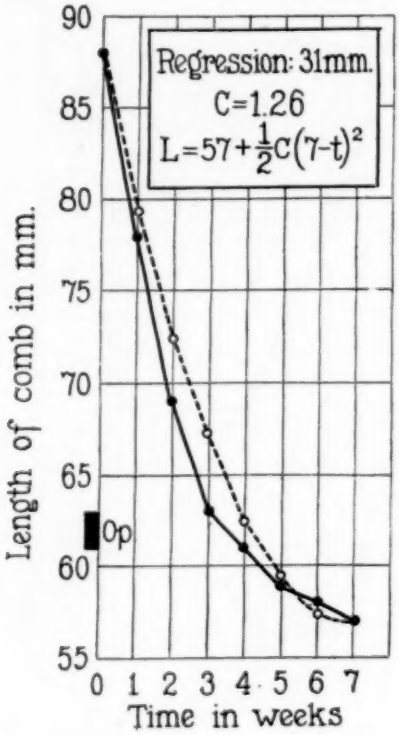

FIG. 6.

We have also plotted the weight curve, as in Experiment 2; there is the same independence as previously concerning the evolution of the comb and the general nutrition.

Comparison of the theoretical and the observed curves shows the coincidence to be as good as possible. Furthermore, if the curve represents the facts as exactly as possible, it must make extrapolation possible; that is, if we know, in a given instance, a portion of the curve

sufficient for determining its constants, we must be able to calculate the other points of the curve beyond those derived from our experimental data. The calculation made on the basis of the measurements on Cock 6 (which died before regression was complete) leads us to conclude that such is the case.

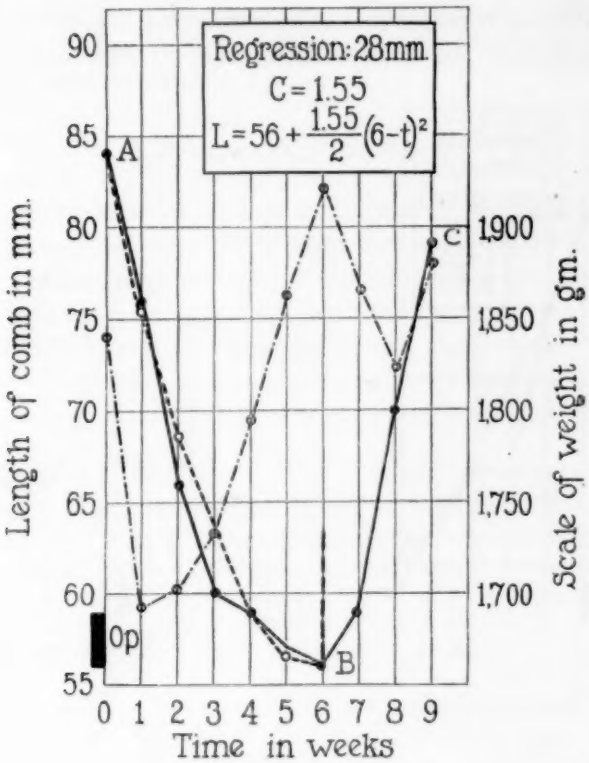

Fig. 7.

Experiment 6.—Cock, Beauceronne, about 6 months old. Secondary sex characters in course of development. Comb and barbules scarlet. Sex insticts present; crowing. Completely castrated Jan. 18, 1912; died Feb. 18 in a state of cachexia (Fig. 8).

Having made five measurements, we know five points of the curve; three are sufficient for the calculation of l, C, θ. Utilizing these, which correspond to 0, 1, and 4 weeks, we find:

$$l = 56.5$$
$$C = 0.5$$
$$\theta = 10.5$$

The value found for l in this case is the same as that furnished by the subjects of the other experiments; the duration of regression is a little long, but not improbable.

The individual constant C is a measure of the negative acceleration of the regression. In order to determine to what factor it corre-

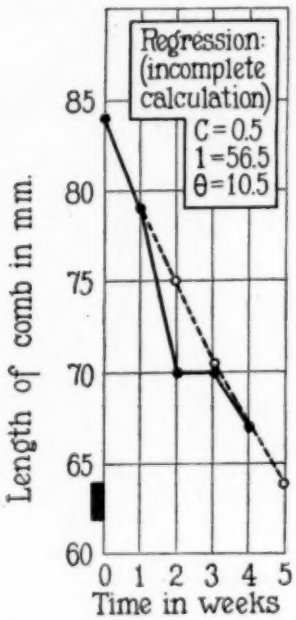

FIG. 8.

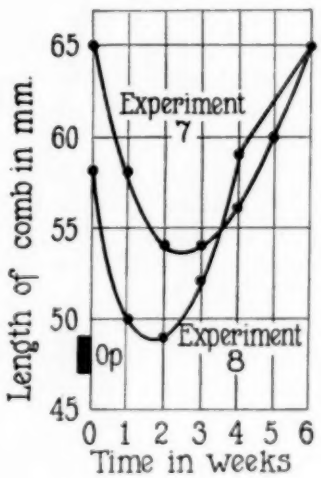

FIG. 9.

sponds, let us note that the abrupt beginning of the curve indicates that the action of the testicle does not survive castration. Under these conditions, the constant C appears to us to be related exclusively to the properties of the erectile tissue. For this reason we have called it the *constant of regression*. On its value and on that of the total decline depends the duration of regression.

Values found.						
C.....	1.55	1.27	1.26	0.72	0.64	0.5
θ.....	6.0	7.0	7.0	12.0	9.0	10.5

It would be interesting to determine the variation with age of the total decline, the constant of regression C, and also the time θ. It is possible that C diminishes with age, while θ increases. But the number of experiments at our disposal do not permit us to affirm it. At all events, by the same method of reasoning, we may regard as established from now on the fact that C and θ are bound to each other by a simple relation. To demonstrate this point, we shall base our reasoning on the figures derived from our experiments on castration followed by transplantation.

Intrapuberal Castration.

The following observations have no bearing on the cocks which had acquired or nearly acquired their secondary sex characters, but upon those birds which were castrated at the time when the development of these characters was beginning (intrapuberal castration). As the diminution in the comb is then slight, one might expect to find a slow regression similar to that which characterized the end of the regression period in the previously observed birds the experiment confirms this hypothesis.

Experiment 7.—Cock, mixed breed, hatched at the Physiological Station in Aug., 1911. Castrated Jan. 22, 1912. Received testicular transplantations at the time of castration. For 3 weeks the comb regressed; then its development recommenced under the influence of the transplantations. The regression and even the resumption of development have a clearly parabolic curve (Fig. 9).

We had calculated C, θ, and l starting from the formula

$$L = l + \tfrac{1}{2}C(\theta - t)^2$$

obtaining successively

$$t = \begin{cases} 0 \\ 2 \\ 4 \end{cases} \qquad L = \begin{cases} 65 \\ 54 \\ 56 \end{cases}$$

whence

$$\begin{aligned} C &= 3.25 \\ \theta &= 2.7 \\ l &= 53.2 \end{aligned}$$

Experiment 8.—Cock, same conditions of experiment and same succession of observations as in the preceding case (Fig. 9). The calculations are the same as in Experiment 7, according to the general formula

$$t = \begin{cases} 0 \\ 2 \\ 4 \end{cases} \qquad L = \begin{cases} 58 \\ 49 \\ 59 \end{cases}$$

whence

$$C = 4.75$$
$$\theta = 1.9$$
$$l = 48.9$$

In both cases, the regression due to intrapuberal castration began immediately after castration, as in normal subjects. Furthermore, although the mass of tissue before regression was much smaller, *the decline began with the same rapidity as in adults*: the diminution in length of the comb was 7 mm. (Experiment 7) and 8 mm. (Experiment 8) during the 1st week. That the length attained at the end of regression was much less than in the preceding cases is explained by the fact that the comb of a castrated fowl of the same age has not yet attained its final length. As to the duration of regression, it is much shorter than in the preceding experiments, now that the constant C is much smaller.

SUMMARY.

The discrepancy in the relative variation of C and of θ led us to examine more closely the velocity of regression at the beginning in all the cases. At a given point of the curve, the velocity is furnished by the differential quotient of the length with reference to the time:

$$v = \frac{dL}{dt} = -C\theta + Ct$$

At the beginning of regression, that is to say, at the time 0

$$v_0 = -C\theta$$

We have tabulated the corresponding numerical values in the various instances:

Experiment No.	C	θ	$-C\theta$
8	4.75	1.95	−9.4
7	3.25	2.7	−8.7
5	1.55	6.0	−9.3
2	1.27	7.0	−8.9
4	1.26	7.0	−8.8
1	0.72	12.0	−8.6
3	0.64	9.0	−5.8 (Species with small comb.)
♂	0.5	10.5	−5.2 (Extrapolation.)

Although there is not absolute equality among the figures of the last column, one cannot fail to be struck by the fact that there is very little difference; in all instances they diverge much less than those of the first two columns, in which the variation is from 0.5 to 4.75 and from 1.95 to 12.0. We must admit, therefore, within rather wide limits, the constancy of the product of the time of regression and the constant C, whether the castration is intrapuberal or postpuberal.

Geometrically, this result is represented by the constancy of the angle of the ordinate and the tangent to the parabola at the point of departure of the regression curve. Furthermore, it follows that the numerical law is represented not only by a parabola, but more exactly by segments of homothetic parabolas—an unexpected generalization, which gives a remarkable unity to the law with which it is concerned.

CONCLUSIONS.

1. Postpuberal castration is followed in cocks by a regression of the comb; this organ acquires in a few weeks the aspect and dimensions which characterize similar organs of cocks castrated before puberty.

2. The diminution in the length of the comb is effected according to a simple law, represented geometrically by a segment of a parabola, algebraically by the equation

$$L = l + \tfrac{1}{2}C(\theta - t)^2$$

3. Intrapuberal castration demonstrates that during the development of the comb C and θ have values very different from those which are furnished by adults, but the product $C\theta$ is constant.

4. This constancy indicates that the diverse curves are represented by similar segments of a parabola.

PHOTOCHEMISTRY OF VISUAL PURPLE.

II. The Effect of Temperature on the Bleaching of Visual Purple by Light.

By SELIG HECHT.

(*From the Physiological Laboratory, College of Medicine, Creighton University, Omaha.*)

(Received for publication, September 23, 1920.)

I.

1. The determination of the influence of temperature on the velocity of a photochemical reaction is always of importance. This is particularly true when the mechanism of the reaction is unknown, as in the present instance of the bleaching of visual purple.

The usefulness of determining the temperature coefficient lies in this. Photochemical reactions have low temperature coefficients,—near unity (Sheppard, p. 304), though exceptions are known. Ordinary chemical reactions, however, have temperature coefficients which are much higher, between 2 and 3 or even more. The order of magnitude of the temperature coefficient may therefore give us some notion of the degree of complexity of a photosensitive reaction. A value of Q_{10} near unity may be taken to indicate that the reaction probably consists of a simple, direct photochemical transformation. On the other hand, a high value for Q_{10} shows most probably that the photochemical reaction proper is only one in a series of reactions which makes up the photosensitive process. The associated chemical reactions, because of their high temperature coefficients, raise the temperature coefficient of the reaction as a whole more nearly to their own level.

An example of just such a condition is the high temperature coefficient ($Q_{10} = 2.5$) of the photosensory process in *Mya*. On analysis (Hecht, 1918–19, *a*; 1919–20, *b*) a series of at least three reactions has been shown to exist, of which the photochemical proper is only the first. This has a temperature coefficient of 1.06 when investi-

gated independently; the values for the temperature coefficients of the other reactions are 2.5 (μ = 19,680) and 13.5 (μ = 48,500) respectively. An analysis of a similar hypothetical condition has been made by Osterhout (1917) which shows the relation of the total temperature coefficient of a process to the temperature coefficients of the individual reactions composing it.

2. In the first paper of this series (Hecht, 1920–21, *c*) it was shown that visual purple solution, so prepared as to give an irreversible photochemical reaction, is bleached by light according to the kinetics of a monomolecular reaction. This in itself would seem to indicate that the reaction as a whole is a simple photochemical transformation.

Experiments with intermittent as compared with constant illumination supported this conclusion. Within the limits of experimental error it was not possible to demonstrate the presence of any initial induction period or of an after effect in the course of the reaction. The presence of either or both of these irregularities frequently bespeaks a complexity in the make-up of the reaction (Mellor, 1904, p. 116).

The evidence therefore indicates that the bleaching of visual purple is a comparatively simple process. This is further confirmed by the following experiments on the effect of temperature.

II.

Visual purple solutions are prepared from the dark-adapted retinas of frogs by the technique previously described.[1] The changes produced by light are followed colorimetrically by comparison with concentration standards. These are made by combining bleached and unbleached visual purple in varying proportions so as to give a series changing by 10 per cent steps. The experimental solutions and the standards are manipulated in capillary tubes arranged to give equal depths of solution. For the composition and preparation of the standards and for the methods generally, the reader is referred to the previous article in these studies (Hecht, 1920–21, *c*) where full details will be found.

[1] In making solutions of visual purple, pure bile salts must be used. I am indebted to Dr. S. Morgulis for furnishing me with a supply of these salts purified by himself.

In order to control the temperature of the exposed solution the capillary tube containing it is kept in a water bath. This is a cylindrical glass vessel, 12 cm. in diameter, and of 900 cc. capacity. The temperature of the bath is maintained constant by the addition of water at a higher or a lower temperature from a nearby reservoir. In this way the temperature is easily kept within 1°C. Such a bath is accurate enough for our purposes, as will presently be evident.

The exposure tube of visual purple is immersed, attached to the rotating apparatus, and kept in the dark for 5 minutes for its contents to reach the temperature of the bath. This is more than enough when we recall that a thermometer having a larger diameter than the capillary tube comes into thermal equilibrium in much less time. After 5 minutes the shutter is opened, and the light allowed to act on the solution, which is rotated in the usual manner. After the proper exposure, the light is shut off, and the concentration of the exposed solution determined. As in the previously reported work, readings are made to the nearest 5 per cent. The tube is then returned to the bath, allowed 5 minutes in the dark to come to the temperature of the bath, and again exposed for an interval. The concentration is again determined, and the process repeated until the solution is fully bleached.

The source of illumination is a 250 watt, concentrated-filament, stereopticon Mazda lamp run on the ordinary lighting circuit. The intensity used throughout these experiments on temperature effects is 50 meter candles. At this intensity bleaching is complete in half an hour.

III.

A number of preliminary experiments showed definitely that the effect of temperature on the velocity of the bleaching process is very small indeed. In this I can confirm the roughly quantitative, early work of Kühne (1879). For example, in a series of three experiments performed, one at 7°C., another at 23°C., and a third at 36°C., the velocity constants for the bleaching process were 0.038, 0.041, and 0.039 respectively. This gives a value for the temperature coefficient as $Q_{10} = 1.00$. The same value for Q_{10} was found for a set of experiments in which the velocity of decomposition was determined

at five temperatures covering the same interval as the previous three. However, in two other series of experiments performed at three temperatures as before, I obtained a value for $Q_{10} = 1.15$.

These four sets of preliminary experiments were carried out by the use of a single tube of visual purple for each temperature. Individual experimental errors may thus possibly account for the differences between the first two and the last two sets of experiments. Therefore, in order to be thoroughly certain of the results, I performed two sets of experiments in which three tubes of visual purple

TABLE I.

Temperature and Bleaching of Visual Purple.

Temperature.	$k=\frac{1}{t}\log\frac{a}{a-x}$	
	Series 1.	Series 2.
°C.		
5.2	0.039	0.031
20.0	0.035	0.031
36.1	0.036	0.033

were bleached simultaneously at each temperature. The velocity constants for the two series are given in Table I together with the temperatures. Each figure is the average of three experiments which agreed among themselves in a way similar to the figures previously quoted for one preliminary experiment.

IV.

1. From these results it will be seen that the change in the velocity constant over a range of 30 degrees is practically *nil.* Graphically this is demonstrated by Fig. 1, which presents the details of the second series of Table I. The rectangles are the averages of the experimental findings at the different temperatures, whereas the curve is the isotherm of a monomolecular reaction

$$k = \frac{1}{t}\log\frac{a}{a-x}$$

in which the velocity constant has the average value of $k = 0.032$. It is apparent that the points at all temperatures fit the single curve as well as can be expected.

It may therefore be concluded that the temperature coefficient for 10°C. for the bleaching of visual purple by light is practically 1.00. This corroborates our idea that the bleaching reaction is a simple photochemical transformation, consisting most probably of a single reaction.

2. There is an additional point to be made with regard to the magnitude of the temperature coefficient. In any photochemical

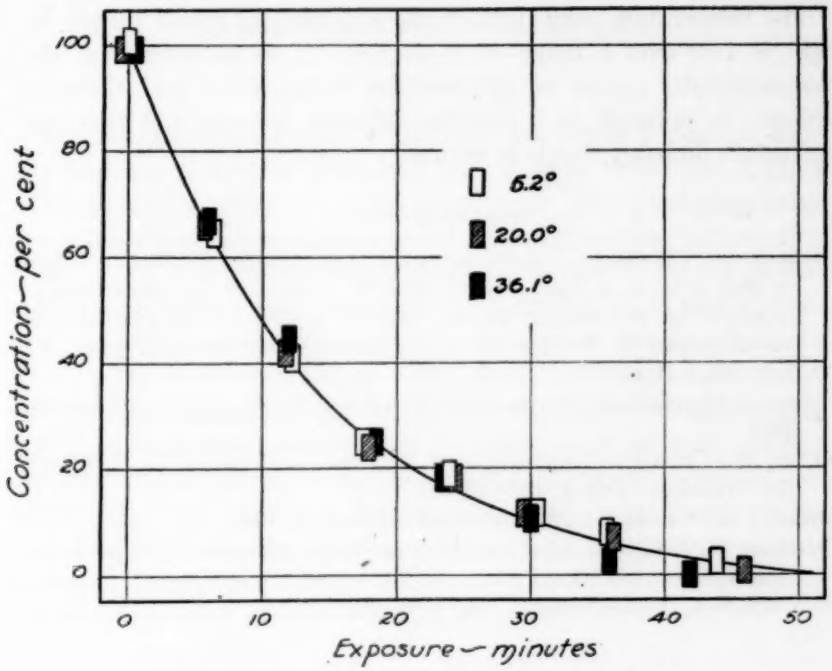

FIG. 1. Temperature and the velocity of photochemical decomposition of visual purple. The curve is a monomolecular isotherm; the rectangles are the experimental results of one set of experiments.

reaction there is always the possibility that the reaction proper takes place with a practically instantaneous velocity at the walls of the vessel, and that the kinetics of the reaction really represent the diffusion velocity of the photosensitive substance to the place of reaction.

The temperature coefficient of diffusion velocities, though lower than those of ordinary chemical reactions, is usually higher than 1.00,

—generally near 1.3 (Höber, 1914, p. 708). The fact that the temperature coefficient of visual purple bleaching is 1.00, combined with the other evidence already at hand, would therefore indicate that the kinetics of the reaction represent a real chemical occurrence rather than a process of diffusion.

SUMMARY.

The temperature coefficient of the bleaching of visual purple by light is 1.00 over a range of 30 degrees. This indicates that the monomolecular course of the reaction represents a real chemical process, as opposed to a possible diffusion process, and that the reaction is probably simple in nature.

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THE AMPHOTERIC PROPERTIES OF SOME AMINO-ACIDS AND PEPTIDES.

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INTRODUCTION.

The change in the hydrogen ion concentration of a solution upon the addition of acid or alkali has been used to follow the changes characteristic of certain dissolved substances. Thus, the progressive neutralization of polybasic acids and of polyacid bases has been studied in this way.[1] Amphoteric electrolytes lend themselves readily to this method of investigation. Recently, the results obtained with a number of proteins have been presented in the form of titration curves in which amounts of standard acid and alkali were plotted along one axis and the resulting pH values of the protein solution along the other axis.[2] Such titration curves may be expected to be more or less characteristic for the acid- and base-combining properties or groups of such substances. In view of the importance and value of such studies in protein chemistry, the titration curves of some of the simple amino-acids and dipeptides are presented here as a necessary preliminary to a more satisfactory understanding of the more complex bodies.

Experimental Methods.

The following substances were studied; glycine, alanine, α-amino-butyric acid, leucine, glycyl-glycine, alanyl-glycine, and alanyl-alanine. Data were obtained also for solutions of sodium chloride, acetone,

[1] Hildebrand, J. H., *J. Am. Chem. Soc.*, 1913, xxxv, 847.

[2] *Cf.* Cohn, E. J., Gross, J., and Johnson, O. C., *J. Gen. Physiol.*, 1919–20, ii, 145. Cohn, E. J., Wolbach, S. B., Henderson, L. J., and Cathcart, P. H., *J. Gen. Physiol.*, 1918–19, i, 221. Sörensen, S. P. L., *Compt. rend. lab. Carlsberg*, 1915–17, xii. Clark, W. M., The determination of hydrogen ions, Baltimore, 1920, especially pp. 34 and 211, for general discussion and references.

acetamide, urea, acetic acid, and aceturic acid. The substances were prepared by the usual methods and showed satisfactory states of purity except for the alanyl-alanine, for which the nitrogen content was found to be 1 per cent less than the calculated value. Attempts at purification did not remedy this, so that evidently an impurity, possibly alanine, was present.

The solutions studied were prepared so that 1 liter of the final mixture contained in every case 0.05 gram molecule of the amino-acid or peptide or other substance, and 0.05 gram molecule of sodium chloride, while the amounts of hydrochloric acid or sodium hydroxide (0.1 N) added were varied. The water used was distilled first from sulfuric acid, then from alkaline permanganate, and condensed in a block tin condenser.

The concentration of the ampholyte or other substance and of the sodium chloride in the final volume (50 cc. as made up and used) was the same in all cases. The only variable was the amount of acid or alkali which was added, or the ratio between this and the concentration of solute.

The hydrogen ion concentrations of the solutions were determined by electromotive force measurements using calomel electrodes with 0.1 M potassium chloride solution, saturated potassium chloride bridge, unknown solution with hydrogen electrode (platinum or gold coated with palladium black), and a potentiometer of Leeds and Northrup type "K" in conjunction with a type "H" galvanometer with a loading coil to decrease its period. Cells of the rocking type described by Clark[3] were used with the modification of having an adapter of about 25 cc. capacity fused on the inlet tube.[4] Hydrogen was obtained compressed in cylinders and was purified by passage through (electrically) heated copper gauze and then bubbled through water.

The customary precautions in purifying the materials, and standardizing the various parts of the apparatus and the solutions were taken. The hydrogen ion concentrations in terms of pH were calculated from the experimental measurements by the formula[5]

[3] Clark, W. M., *J. Biol. Chem.*, 1915, xxiii, 475.

[4] The writers desire to thank Professor F. S. Lee of the Department of Physiology of Columbia University for presenting to them the excellent rocking apparatus used in this work.

[5] Sörensen, S. P. L., *Compt. rend. lab. Carlsberg*, 1909, viii, 22, 29.

$$\text{pH} = \frac{\pi - 0.3377}{0.0577 + ((t - 18)\,0.0002)}$$

in which π = reading in volts; 0.3377 = potential of calomel electrode; and $0.0577 + ((t - 18)0.0002)$ = correction factor for temperature $t°$, as a thermostat was not used.

In a number of cases the hydrogen ion concentrations were also determined colorimetrically. Where this was done, the indicators and standard solutions described by Clark and Lubs were used.[6]

The isoelectric points of the various ampholytes were also determined by the method described by Michaelis[7] and developed by Sörensen.[8] This consists of adding the pure ampholyte solution to a series of solutions of progressively changing hydrogen ion concentration containing the indicator and noting where no change in color occurred on mixing the unknown ampholyte solution and standard solution.

Experimental Results.

The results obtained for the pH values for the solutions of the various substances in the presence of acid and alkali are given in Table I. Although not stated in the table, it may be recalled that all the solutions contained sodium chloride of the concentration 0.05 M. Column 1 headed "Water" contained no added solute but the sodium chloride and acid or alkali. The headings of the remaining columns indicate the solute present, 0.05 M in every case.

The titration curves plotted from these results are given (Figs. 1, 2, and 3). The pH values are given as abscissæ, and as ordinates are shown the number of cc. of 0.1 N hydrochloric acid or sodium hydroxide added to 25 cc. of 0.1 M solution of the indicated solute (also 0.1 M with respect to sodium chloride), and the whole diluted to 50 cc. The numbering of the curves corresponds to the numbering of the columns in Table I. Since the results of the amino-acids are so nearly alike, only one curve (Curve 6, Fig. 2) is given. Similarly for the dipeptides, only one curve (Curve 10, Fig. 3) is presented.

[6] Clark, W. M., and Lubs, H. A., *J. Bact.*, 1917, ii, 1, 109, 191.

[7] Michaelis, L., *Biochem. Z.*, 1912, xlvii, 251. Hasselbalch, K. A., *Biochem. Z.*, 1916, lxxviii, 129.

[8] Sörensen,[2] p. 150.

TABLE I.

Milliequivalents of HCl or NaOH per liter of solution containing 50 milliequivalents of solute.	(1) Water.	(2) Urea.	(3) Acetamide.	(4) Acetone.	(5) Acetic acid.	(6) Glycine.	(7) Alanine.	(8) α-Aminobutyric acid.	(9) Leucine.	(10) Glycylglycine.	(11) Alanylglycine.	(12) Alanylalanine.	(13) Aceturic acid.
						HCl							
50	1.32	1.29	1.29	1.29	1.27	1.94	1.84	1.75	1.85	2.26	2.21	2.18	1.23
44						2.02	1.95	1.85	1.97	2.48			
40	1.45	1.39	1.39	1.39		2.11	2.05	1.97	2.05	2.65	2.74	2.55	
34						2.26	2.18	2.08	2.21	2.89	2.83		
30	1.55	1.50	1.50	1.50	1.49	2.32	2.32	2.25	2.30	3.00	2.97	2.75	1.46
24						2.51	2.49	2.42	2.44	3.29	3.15	3.09	
20	1.68	1.68	1.69	1.67		2.62	2.61	2.54	2.55	3.34	3.28	3.21	
18						2.70	2.68	2.62	2.63	3.42	3.39		
14	1.85					2.85	2.82	2.76	2.77	3.58	3.53	3.59	
10	2.07	1.97	2.00	1.97	1.95	3.02	3.01	2.92	2.94	3.75	3.71	3.83	1.88
8						3.13	3.11	3.04	3.07	3.88	3.84	3.90	
6	2.23				2.09	3.28	3.25	3.16	3.21	4.02	3.99	4.03	2.03
4						3.46	3.43	3.35	3.42	4.21	4.19	4.35	
2	2.71				2.52	3.75	3.72	3.63	3.71	4.65	4.42	4.58	2.24
1	2.96					4.06	4.04	3.92	4.05	4.82	4.74	5.16	

NaOH													
0	4.31	4.82	5.00	4.31	2.88	5.39	5.34	4.75	6.12	5.64	5.21	5.59	2.36
1	10.31			10.63		8.04	8.02		7.89	6.58	6.43	6.48	2.39
2	10.98			11.05	3.25	8.34	8.35	(7.14)	8.19	6.93	6.76	6.84	2.49
4						8.70	8.71	8.48	8.53	7.24	7.10	7.17	
6	11.64			11.70	3.67	8.83	8.85	8.73	8.71	7.45	7.32	7.33	2.69
8						9.07	9.00	8.94	8.86	7.61	7.53	7.50	
10	11.90	11.92	11.88	11.93	3.93	9.16	9.13	9.10	9.01	7.71	7.59	7.61	2.90
14	12.04					9.34	9.31	9.32	9.19	7.88	7.83	7.80	
18						9.50	9.47	9.51	9.35	8.03	7.93	7.93	
20	12.20	12.23	12.21	12.27	4.34	9.55	9.54	9.57	9.43	8.11	8.01	8.01	3.28
24						9.71	9.78	9.73	9.56	8.26	8.23	8.16	
30	12.38	12.40	12.34	12.43	4.69	9.89	9.98	9.92	9.77	8.43	8.39	8.36	3.64
34						10.02	10.12	10.08	9.89	8.61	8.55	8.53	
40	12.50	12.51	12.45	12.59	5.10	10.28	10.37	10.27	10.12	8.85	8.75	8.77	4.12
44						10.49	10.58	10.46	10.35	9.08	9.05	9.08	4.50
50	12.59	12.54	12.65	12.64	6.51	10.86	10.93	10.88	10.81	9.78	9.81	10.23	(10.05)

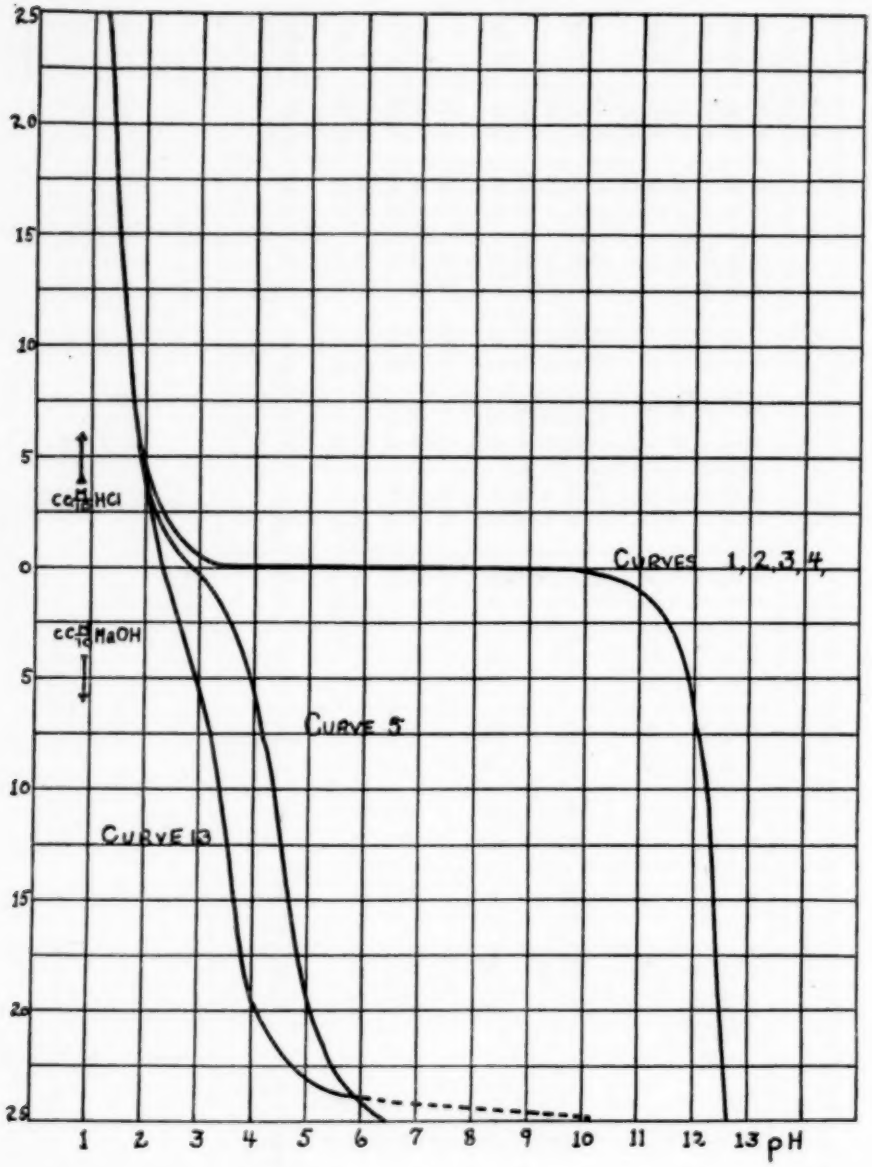

FIG. 1. Titration curves of water (1), urea (2), acetamide (3), acetone (4), acetic acid (5), and aceturic acid (13).

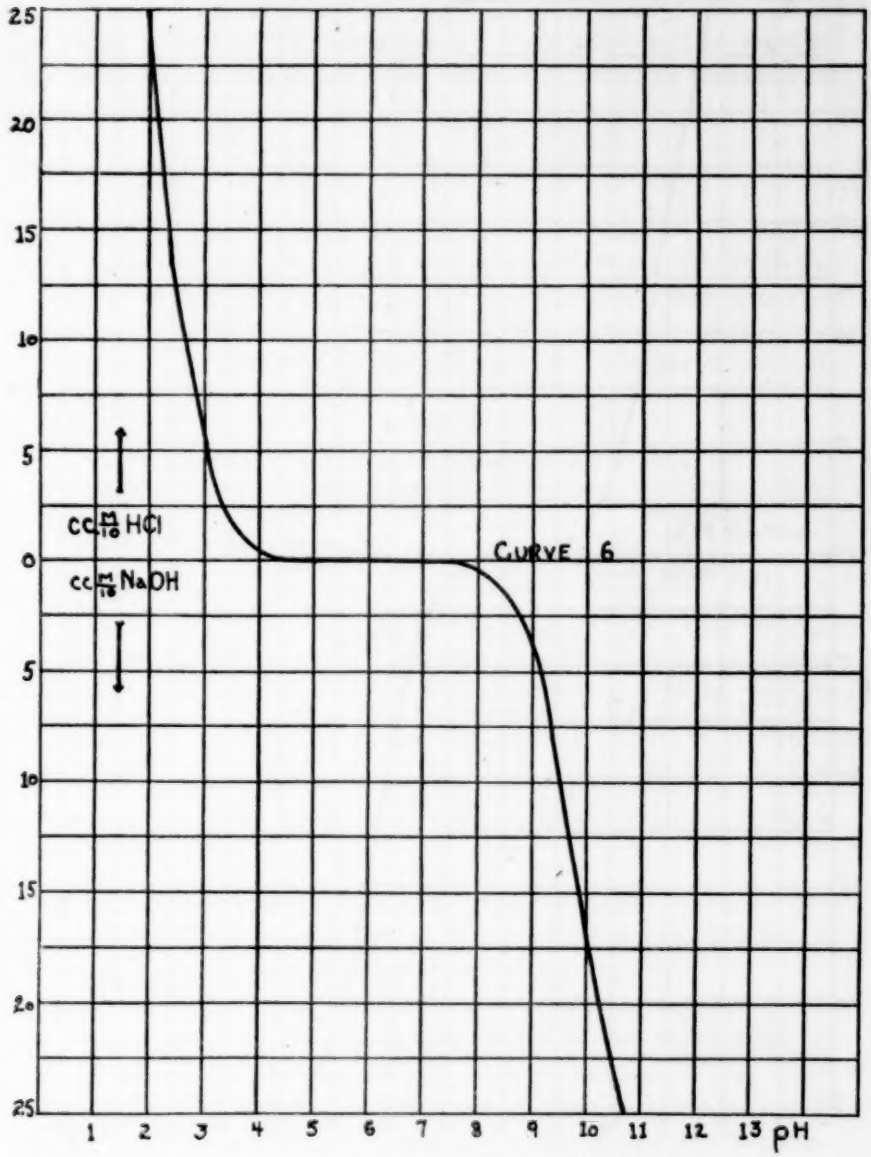

FIG. 2. Titration curve of glycine. Practically identical with titration curves of alanine, α-amino-butyric acid, and leucine.

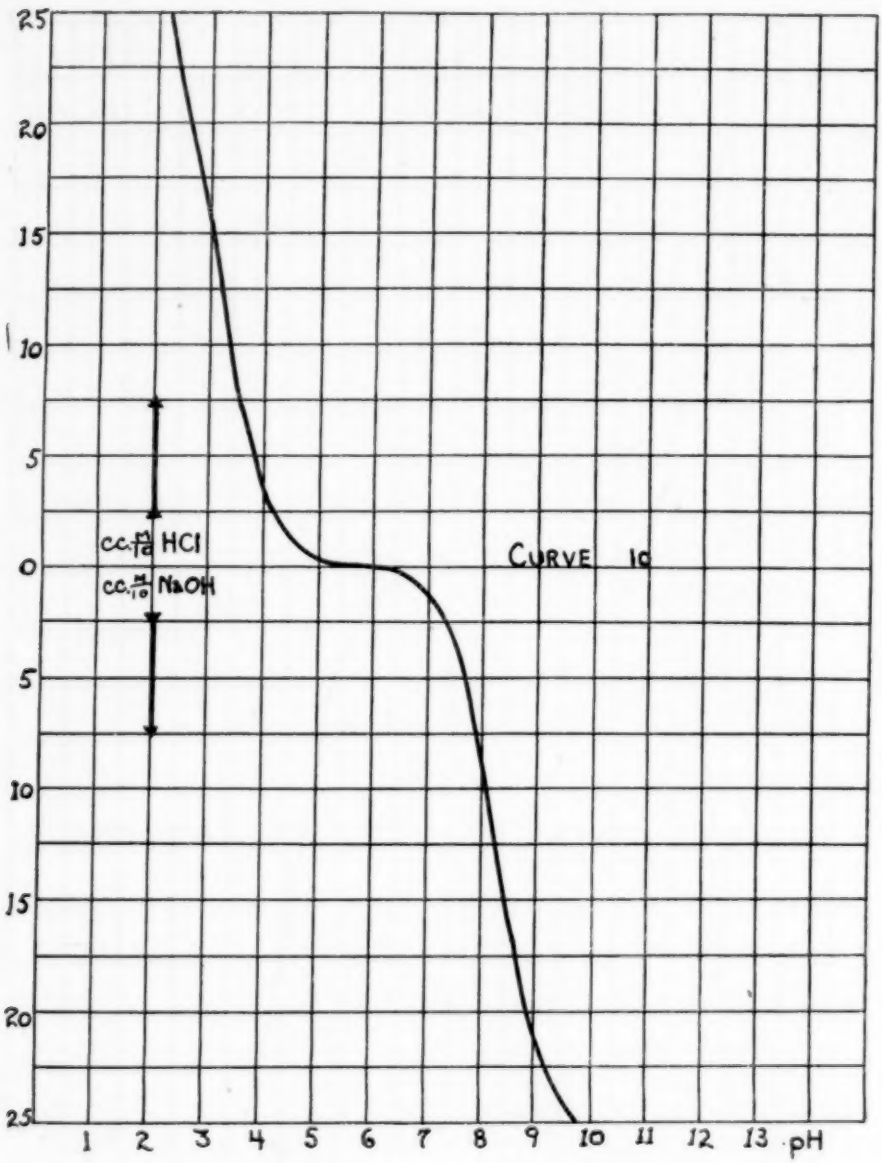

FIG. 3. Titration curve of glycyl-glycine. Practically identical with titration curves of alanyl-glycine and alanyl-alanine.

The values for the isoelectric points of some of the substances are given in Table II. The significance of these points will be discussed in detail later in this paper. The values calculated by means of the expression

$$I = \sqrt{k_w \frac{k_a}{k_b}}$$

in which k_a and k_b represent the ionization constants of the ampholyte substance acting as an acid and as a base, and k_w the ionization constant of water (very nearly 10^{-14} at the temperatures used) are given and also the values determined experimentally by means of the potentiometer (values from Table I), and by the indicator methods.

TABLE II.

Isoelectric Points of Some Amino-Acids and Peptides.

	k_a*	k_b*	Isoelectric points.		
			Calculated.	Potentiometer.	Indicator.
			pH	*pH*	*pH*
Glycine	1.8×10^{-10}	2.7×10^{-12}	6.1	5.4	6.0
Alanine	1.9×10^{-10}	5.1×10^{-12}	6.2	5.3	6.15
Leucine	1.8×10^{-10}	2.5×10^{-12}	6.1	6.1	7.0
Glycyl-glycine	1.8×10^{-8}	2.0×10^{-11}	5.5	5.6	5.5
Alanyl-glycine	1.8×10^{-8}	2.0×10^{-11}	5.5	5.2	5.15
Alanyl-alanine				5.6	6.15

* Winkelblech, K., *Z. physik. Chem.*, 1901, xxxvi, 546. Euler, H., *Z. physiol. Chem.*, 1907, li, 219.

Before discussing these results, a number of points connected with the experimental measurements will be taken up briefly; first for the results given in Table I, then for those in Table II.

Recently it was stated[9] that "There is hardly any use attempting the measurement of unbuffered solutions, if indeed there would be any significance to the measurement were it accurate." This statement apparently necessitates further explanation, possibly basing the definition of the term buffer in some way upon the slope of the titration curve. Otherwise, solutions of hydrochloric acid, etc., would be

[9] Clark,[2] p. 184. *Cf.* also a similar statement on p. 34.

included among those for which accurate determinations are not possible.

This raises the question of possible inaccuracies in the determinations. Aside from such questions as the presence of small amounts of impurities at or near the isoelectric points of ampholyte solutions where the buffer action is found to be small,[10] certain points connected with the relative accuracies of the chemical and electrical measurements are involved. Comparing, for example, the determination of the hydrogen ion concentration of 0.1 N hydrochloric acid (pH approximately 1.0) and of 0.1 N sodium hydroxide (pH approximately 13.0), the electrical measurement is much more sensitive for the latter. The component determined in both cases is the hydrogen ion. In the preparation of the solutions, the alkaline solution is more readily influenced or changed (perhaps due to the accidental nature of the surroundings) as regards its hydrogen ion concentration. Alkaline solutions may therefore require the presence of buffer mixtures in order to obtain constant and reproducible results under conditions with which analogous acid solutions apparently give accurate values with no buffer present.

The presence of sodium chloride of the given concentration had no appreciable influence on the determinations as shown by the work of Harned[11] and of Fales and Nelson[12] on mixtures of acid and neutral salts, of Sörensen[13] who compared ampholyte solutions with and without salt, and of Tague[14] who measured amino-acid solutions without salt.

Some determinations by others may be compared with the results given here, although in most cases the results are not comparable for a whole series. The solutions with no added solute besides the acid and alkali agree satisfactorily with such results[15] except that Tague appeared to be more successful in excluding carbon dioxide. The results for glycine agreed well with those of Sörensen[13] and of Tague[14] where comparison is possible.

[10] *Cf.* Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 1914, for a careful and systematic discussion of these relations.

[11] Harned, H. S., *J. Am. Chem. Soc.*, 1915, xxxvii, 2460.

[12] Fales, H. A., and Nelson, J. M., *J. Am. Chem. Soc.*, 1915, xxxvii, 2773.

[13] Sörensen, S. P. L., *Compt. rend. lab. Carlsberg*, 1909, viii.

[14] Tague, E. L., *J. Am. Chem. Soc.*, 1920, xlii, 173.

[15] *Cf.* Frary, F. C., and Nietz, A. H., *J. Am. Chem. Soc.*, 1915, xxxvii, 2264.

The results have been presented without introducing the correction used by Tague in his work in which the amount of acid or alkali required to bring the solution without solute to a definite hydrogen ion concentration was subtracted from the amount required with the solute present. This corrected value would give a more nearly accurate measure of the reaction between amino-acid, etc., and acid or alkali. For the results given here, this correction is negligible except for the largest additions of acid or alkali. If desired, the data given in Column 1 of Table I will permit the introduction of these corrections.

With regard to the isoelectric point results given in Table II, the determinations by the potentiometer method, that is the determination of the hydrogen ion concentrations of the solutions with neither acid nor alkali added, cannot lay claim to any great degree of accuracy for the amino-acids since the buffer action is so slight and since a broad zone exists on the curve where traces of acid or alkali may change the results appreciably. With the dipeptides this zone is narrower and the results should be correspondingly more accurate. The indicator method appears to give somewhat more accurate results as fewer manipulations are required. However, too much stress must not be placed upon their exactness.

Discussion of Results.

The data given refer to one concentration (0.05 M) for each substance. This naturally limits for the present the general applicability of the conclusions.

Michaelis[10] and others[16] have developed the theoretical interpretation of such titration curves and applied their conclusions to the results of a number of substances. Among other applications, they have considered the relations to the acid and basic dissociation constants of the ampholyte, to conditions for the smallest and greatest buffer actions, etc. In this paper, the discussion will be limited to questions which have been discussed briefly or not at all by others.

[16] *Cf.* Clark[2] for the general treatment and for complete references.

The following conclusions may be drawn from the results given in Table I and shown in the curves.

(*a*) The results for water, urea, acetamide, and acetone are practically identical. There is therefore at the dilutions used no combination involving the amino groups of urea or acetamide and hydrochloric acid, or enolization of urea, acetamide, or acetone accompanied by reaction with alkali which would be manifested by a change in hydrogen ion concentration.

(*b*) The results for the four amino-acids are nearly the same, showing only minor differences. Thus, on the acid side, alanine and leucine are identical, glycine and α-amino butyric acid differing slightly and in opposite directions; on the alkaline side glycine and alanine are the same differing slightly from the other two. There is a broad zone near the point at which no acid or alkali was added where practically no buffer action is shown.

(*c*) The results for the dipeptides, except for a few apparently minor differences, are strikingly similar throughout.

(*d*) As compared with the amino-acids, there was a very much smaller range where little or no buffer action was shown by the dipeptides. Aside from this, the curves for the two series were practically parallel. The vertical differences between the two sets of curves show the differences in the amounts of acid and of alkali required to bring the substances to the same pH values. Much more acid or alkali was required for the dipeptides than for the amino-acids, the difference being due evidently to the $-CO-NH-$ group of the dipeptides. The differences increase with increasing quantities of acid or alkali added, reach a maximum, and then decrease again. The chemical nature of the $-CO-NH-$ group readily accounts for these properties, acid combining with the $-CO-NH-$ group, alkali bringing about enol-lactim rearrangement and accompanying reaction.

(*e*) Aceturic acid on the acid addition side showed the same results as acetic acid and water. The influence of the highly ionized hydrochloric acid predominated over any effects of the weaker acids. On the alkaline side, the pH values found for aceturic acid were smaller than those for acetic acid with the same amount of alkali. This difference is evidently connected with the presence of the $-CO-NH-$ group and analogous to the difference between dipeptides and amino-

acids. The vertical differences between the two curves also show the extent of base-combining property or enol-lactim tautomerism.

The method of plotting pH values against the amounts of acid and alkali added is also open to question, but it appears to be the best method which is available. Placing the emphasis on the hydrogen ion concentration gives a one-sided chemical perspective, especially for the alkaline solutions. The suggestion of Wherry[17] to use pH 7.0 as the zero point and to calculate values from this as a logarithmic function is not practicable as indicated by Clark.[18] The suggestion may be made to determine a zero point for each substance, this point to be identical with the isoelectric point, and to measure acidity and alkalinity from this point logarithmically in terms similar to the pH scale. This would fix a characteristic property for each substance as the starting point. The practical difficulty lies in the determination of the isoelectric point, as its experimental measurement is probably the least accurate point of most titration curves.

The general chemical nature of amphoteric electrolytes will be taken up briefly here. These substances, depending upon conditions, ionize or react as salts in which the complex ampholyte component acts as the positive constituent or as the negative constituent. Loeb[19] has recently pointed out clearly and convincingly, and has presented considerable experimental evidence to show, that the positive ampholyte ion (of gelatin, for example) is fundamentally different from the same ampholyte as negative ion. The relative acidity and alkalinity of the solution govern these changes, the ionization taking the different courses in more acid or more alkaline solutions (the transition point will be taken up presently). In order to account for the different processes of ionization, it appears to be necessary to assume that the molecule (unionized) is different in the two cases. In order to ionize so that the complex is part of the cation, the molecule must possess a structure different from that of the molecule which ionizes with the complex as part of the anion. This difference in structure may be due to an isomeric rearrangement, possibly tautomeric, to a difference in the action of the solvent involving hydration, or to some

[17] Wherry, E. T., *J. Washington Acad. Sc.*, 1919, ix, 305.

[18] Clark,[2] p. 28.

[19] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 39, 237, 363, 483, 559; 1919–20, ii, 87.

other cause. An equilibrium relation between the two (or more) unionized forms would exist as a rule. This conception complicates somewhat the theoretical treatment of amphoteric electrolytes presented by Bredig,[20] Walker,[21] and others by the addition of an equation representing such an equilibrium and by introducing the concentration of the suitable molecular species in the equations representing the ionization constants. In strongly acid or alkaline solution, however, practically only one form would be present.

The difference in the ionization of the ampholyte is not always brought out clearly. For example, with a protein, the two kinds of ions are sometimes indicated as Na^+ (Protein$^-$) and (Protein$^+$) Cl^-. As a matter of fact, rarely, if ever, do the ions (Protein$^-$) and (Protein$^+$) have even the same chemical composition. This may be indicated by taking the simple case of glycine. Here, the ions are $Cl^-(NH_3CH_2CO_2H)^+$ or $Cl^-(C_2H_6O_2N)^+$, and $(NH_2CH_2CO_2)^-Na^+$ or $(C_2H_4O_2N)^-Na^+$, or $(HONH_3CH_2CO_2)^-Na^+$ or $(C_2H_6O_3N)^-Na^+$, omitting possible hydration of the ions.

The consideration of the transition point spoken of in connection with the different kinds of ionization is a more difficult one. It can best be treated from the side of the isoelectric points of the amphoteric substances.

The definitions of isoelectric point given by various workers have been contradictory at times. The most satisfactory definition appears to be that hydrogen ion concentration at which the properties such as electrical conductivity, viscosity, solubility, etc., when studied over extended ranges of acidity in moderately dilute solutions, show a point of inflexion.[19] Chemically this would be interpreted as stating that in more acid solutions the substance ionizes with the complex as part of the cation, in more alkaline solutions, as part of the anion. At the isoelectric point combination with added acid or base and accompanying ionization is a minimum, or the substance is in a maximum uncombined state. The method of cataphoresis in which the hydrogen ion concentration is determined at which the ampholyte does not migrate in solution or migrates in both directions under the influence

[20] Bredig, G., *Z. Elektrochem.*, 1899–1900, vi, 33.

[21] Walker, J., *Proc. Roy. Soc. London, Series B*, 1904, lxxiii, 155; 1905, lxxiv, 271; *Z. physik. Chem.*, 1904, xlix, 82; 1905, li, 706.

of an electric current has been used to determine the isoelectric points of a number of proteins and other complex bodies.[10, 22]

The equation for calculating the isoelectric point of an amphoteric electrolyte

$$I = \sqrt{\frac{k_a}{k_b} k_w}$$

was deduced by Michaelis and Mostynski.[23] Their deduction does not appear to be altogether clear in so far as the assumptions involved are concerned. The following deduction brings out perhaps more satisfactorily these assumptions. It includes the view that the concentration of the unionized molecule in each case is given by the total number of molecules, not separate molecular species as indicated above.

$$k_a\,(\mathrm{HAOH}) = (\mathrm{H^+})\,(\mathrm{AOH^-})$$

$$k_b\,(\mathrm{HAOH}) = (\mathrm{OH^-})\,(\mathrm{HA^+})$$

$$\frac{k_a}{k_b} = \frac{(\mathrm{H^+})(\mathrm{AOH^-})}{(\mathrm{OH^-})(\mathrm{HA^+})}$$

$$= \frac{(\mathrm{H^+})^2}{k_w} \cdot \frac{(\mathrm{AOH^-})}{(\mathrm{HA^+})}$$

If

$$(\mathrm{AOH^-}) = (\mathrm{HA^+})$$

$$(\mathrm{H^+}) = \sqrt{\frac{k_a}{k_b} k_w} \qquad (1)$$

This derivation assumes the equalities of (HAOH) in the two ionization equations and of ($\mathrm{AOH^-}$) and ($\mathrm{HA^+}$). In the Michaelis and Mostynski original deduction, the assumptions were stated to be the equality of the complex anion and cation concentrations and a minimum total ionization of the ampholyte.

The following deduction involves the view of different molecular species ionizing as acid and as basic salts.

[22] Hardy, W. B., *J. Physiol.*, 1898, xxiv, 288. Cohn, Gross, and Johnson,[2] p. 149.

[23] Michaelis, L., and Mostynski, B., *Biochem. Z.*, 1910, xxiv, 79.

$$k_a\,(\mathrm{HAOH}) = (\mathrm{H^+})\,(\mathrm{AOH^-})$$

$$k_b\,(\mathrm{HA_1OH}) = (\mathrm{OH^-})\,(\mathrm{HA_1^+})$$

$$\frac{k_a}{k_b} = \frac{(\mathrm{H^+})\,(\mathrm{AOH^-})\,(\mathrm{HA_1OH})}{(\mathrm{OH})\,(\mathrm{HA_1^+})\,(\mathrm{HAOH})}$$

$$= \frac{(\mathrm{H^+})^2\,(\mathrm{AOH^-})\,(\mathrm{HA_1OH})}{k_w\,(\mathrm{HA_1^+})\,(\mathrm{HAOH})}$$

$$(\mathrm{H^+}) = \sqrt{\underset{(a)}{\frac{k_a}{k_b}}\,k_w \cdot \underset{(b)}{\frac{(\mathrm{HA_1^+})}{(\mathrm{HA_1OH})}} \cdot \underset{(c)}{\frac{(\mathrm{HAOH})}{(\mathrm{AOH^-})}}} \qquad (2)$$

Equation (2) differs from equation (1) in containing the additional terms (*b*) and (*c*). For the two equations to give the same isoelectric point in any given case either of the following relations must hold (these are fundamentally the same with the terms arranged differently).

$$\frac{(\mathrm{HA^+})}{(\mathrm{AOH^-})} = \frac{(\mathrm{HA_1OH})}{(\mathrm{HAOH})} \qquad (3)$$

$$\frac{(\mathrm{HA_1^+})}{(\mathrm{HA_1OH}) + (\mathrm{HA_1^+})} = \frac{(\mathrm{AOH^-})}{(\mathrm{HAOH}) + (\mathrm{AOH^-})} \qquad (4)$$

From equation (4) it is evident that the degrees of ionization of the two molecular species are the same. This states nothing in regard to the concentrations of the ions or of the molecular species from which they are derived. Also, from equation (3), the ratio of the ampholyte ion concentrations is equal to the ratio of the corresponding unionized molecular species concentrations. That is to say, a greater ionic concentration of the ampholyte as acid species is accompanied by a greater relative concentration of the corresponding unionized molecular species. Obviously, also, equations (1) and (2) are identical if $(\mathrm{HA_1^+}) = (\mathrm{AOH^-})$ and $(\mathrm{HA_1OH}) = (\mathrm{HAOH})$, the assumptions under which equation (1) was deduced.

Equations (1) and (2) do not give the same isoelectric point, if the reciprocal of (*c*) is larger than (*b*), or the ionization as acid is greater than as base. The product of (*b*) and (*c*) will then be less than unity and the value obtained by means of equation (2) will be less than that given by equation (1). The reverse relation holds similarly.

Because of insufficient data, the application of these relations is possible only in isolated cases. For simple substances, including amino-acids, etc., the isoelectric point as defined is probably identical with the hydrogen ion concentration of the pure substance dissolved in water. For more complex substances such as proteins, with a number of different acid- and base-combining groups, there will probably ordinarily be an overlapping of actions. The isoelectric point will then be the hydrogen ion concentration which involves a minimum combination with added acid or alkali, where the protein exists most nearly uncombined. The isoelectric point of a substance obviously shows the relative strengths of the substance acting as an acid and as a base.

Equation (1) requires that the isoelectric point of an ampholyte does not change with change in concentration.[24] Some results with glycine and asparagine show definite if small changes in the hydrogen ion concentrations of solutions of these substances on dilution.[25] The use of equation (2) may help to explain these variations.

The agreement between the isoelectric points calculated by means of equation (1) and those found experimentally is surprisingly close in many cases. For substances such as glycine, etc., where the values of the acid and basic dissociation constants are not far removed from each other and the isoelectric points in the neighborhood of the hydrogen ion concentration of the solvent, this is not unexpected. For a substance like aspartic acid, for which $k_a^{26} = 1.5 \times 10^{-4}$ and $k_b^{26} = 1.2 \times 10^{-12}$, the calculated isoelectric point according to equation (1) is very nearly $(H^+) = 10^{-3}$N. The value found by the indicator method was $(H^+) = 10^{-2.9}$N. This can only mean that there is some sort of compensation with terms (*b*) and (*c*) of equation (2) resulting in the calculated values differing to only minor extents from those given by equation (1). It must be remembered, however, that this compensation is not a necessary conclusion in every case as far as known at present, but that differences may be shown by the two equations.

[24] *Cf.* Tizard, H. T., *J. Chem. Soc.*, 1910, xcvii, 2490.

[25] Quoted by Clark,[2] p. 30, from results of S. P. L. Sörensen.

[26] Winkelblech, K., *Z. physik. Chem.*, 1901, xxxvi, 546. Lundén, H., *Z. physik. Chem.*, 1906. liv. 532; *J. Biol. Chem.*, 1908, iv, 287.

SUMMARY.

The titration curves of solutions of glycine, alanine, α-amino-butyric acid, leucine, glycyl-glycine, alanyl-glycine, alanyl-alanine, acetone, acetamide, urea, acetic acid, and aceturic acid were determined and some of the relations as dependent upon the chemical structures discussed.

The isoelectric points of some of the amphoteric electrolytes were found experimentally. The definition of isoelectric point, its theoretical significance, and method of calculation were considered in some detail.

THE ISOELECTRIC POINT OF RED BLOOD CELLS AND ITS RELATION TO AGGLUTINATION.

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INTRODUCTION.

A number of investigations have been made into the electrical charges carried by bacteria and other cells in watery suspension. The movement observed in cataphoresis is affected by a number of factors, the analysis of which is so difficult that we have at the present time very little information as to the relation between agglutination and the electrical charge of the cells. Cellular suspensions behave in many ways like colloidal solutions, and it is generally understood now that colloidal protein solutions are amphoteric electrolytes. Their colloidal particles carry a negative charge in alkaline reactions and a positive charge in acid reactions; at a definite hydrogen ion concentration there exists no difference in electrical potential between the particles and the medium, so that the particles appear uncharged. This is known as the isoelectric point. At this point a number of physical properties such as solubility, viscosity, and conductivity pass through a minimum. Further, as shown by Loeb,[1] the isoelectric point is a turning point for the chemical change that determines the nature of the ionization. It is to this ionization that the electrical charge is due, so that when ionized as an acid and combined with the cation on the alkaline side of the isoelectric point the protein particle behaves as an anion and moves in the electric field to the anode; when ionized as a base and combined with anion, at reactions more acid than the isoelectric point, the protein becomes part of a complex

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[1] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 39, 237, 363, 483, 559.

cation and moves to the cathode. Chemically, therefore, the protein exists most nearly "pure," least combined with acid or alkali, at the isoelectric point. It is the purpose of the present paper to show the applicability of this conception of the physical and chemical significance of the isoelectric point to the agglutination of red cell suspensions.

The literature pertaining to this subject may be reviewed briefly. The movement of red blood cells in the electric field has been observed by Lillie,[2] by Girard-Mangin and Henri,[3] and by others since. Höber[4] found that the original negative charge became positive on saturation of the solution with CO_2 in the presence of very small amounts of electrolyte, but found that the charge was not reversed if large amounts of salt were present. This effect he referred to the acid character of the CO_2 introduced, and ascribed the reversal of charge to the permeability of the cell wall to anions at the increased hydrogen ion concentration. He noted that ferric and aluminium salts conferred a positive charge upon the cells in all concentrations of NaCl in the solution. Mines[5] found the red blood cells of *Scyllium* negatively charged in the serum. On the addition of strong cerium chloride solution to a suspension of cells the charge becomes uniformly positive and no agglutination occurs; with dilute cerium solution the cells promptly agglutinate. This Mines explains by assuming that in dilute cerium solution only certain of the cells are affected; these cells encounter others still negatively charged and mutual neutralization results, with precipitation of the neutralized cells.

Electric transport of bacteria has been carried out by many investigators. Sears and Jameson,[6] pupils of Zinsser, studied the migration of bacteria at varying degrees of acidity and found that addition of alkali increased their rate of movement while acid lessened it. The fact that protein solutions flocculate most readily near their isoelectric point has suggested that this may be the mechanism of bacterial agglutination, but Beniasch[7] in studying acid agglutination of bacteria

[2] Lillie, R. S., *Am. J. Physiol.*, 1903, viii, 273.
[3] Girard-Mangin, and Henri, V., *Compt. rend. Soc. Biol.*, 1904, lvi, 866.
[4] Höber, R., *Arch. ges. Physiol.*, 1904, ci, 607; 1904, cii, 196.
[5] Mines, G. R., *Kolloid Chem.*, 1911–12, iii, 191, 236.
[6] Sears, H. J.,and Jameson, E., Cataphoresis of Bacteria, San Francisco, 1912.
[7] Beniasch, M., *Z. Immunitätsforsch., Orig.*, 1911, xii, 268.

found that alterations in the hydrogen ion concentration produced no change in the electrical properties of the bacteria; their original negative charge was not neutralized at the optimal point for agglutination and was not reversed by a more acid reaction. Arkwright[8] found, however, two optimal zones for acid agglutination of typhoid bacilli; in the more acid zone (pH 3.0) agglutination of the bacterial bodies is associated with absence of electrical charge. In the less acid zone (pH 4.7 to 3.5) a soluble bacterial protein precipitates and no change in charge is noted.

As to the influence of specific sensitization very little is known. Neisser and Friedemann[9] observed that agglutinin bacteria flocculate in the electric field as a direct result apparently of the electric flow. Michaelis and Davidsohn[10] conclude that specific typhoid agglutination and precipitation are independent of the H concentration, although from their tables for agglutination it appears that there is an optimum for this phenomenon lying between pH 4.6 and 3.7 very close to that of the native bacteria, which is at pH 4.4.

EXPERIMENTAL.

The isoelectric point was determined in the present work by the method of cataphoresis, using a model of U-tube evolved after experiments with many types of apparatus. It is illustrated in Fig. 1. The non-polarizable electrodes consist of zinc rods inserted through perforated rubber stoppers into the long ends of large stop-cock tubes. The lower portion of these tubes and the cock itself are filled with 15 per cent zinc sulfate solution. The upper part of these tubes contains a buffer solution of acetic acid acetate or phosphate mixture. This solution is made up to 10 per cent of saccharose, in order to have a specific gravity less than that of the zinc sulfate and greater than that of isotonic (M/4) saccharose solution alone, to minimize diffusion when the different fluids are in contact. The U-tube itself has an inside diameter of 6 mm. and a total length of 30 cm. It is made in two equal portions, so that after filling the side arms including the

[8] Arkwright, J. A., *Z. Immunitätsforsch., Orig.*, 1914, xxii, 396.

[9] Neisser, M., and Friedemann, U., *Münch. Med. Woch.*, 1904, li, 465, 827.

[10] Michaelis, L., and Davidsohn, H., *Biochem. Z.*, 1912, xlvii, 59.

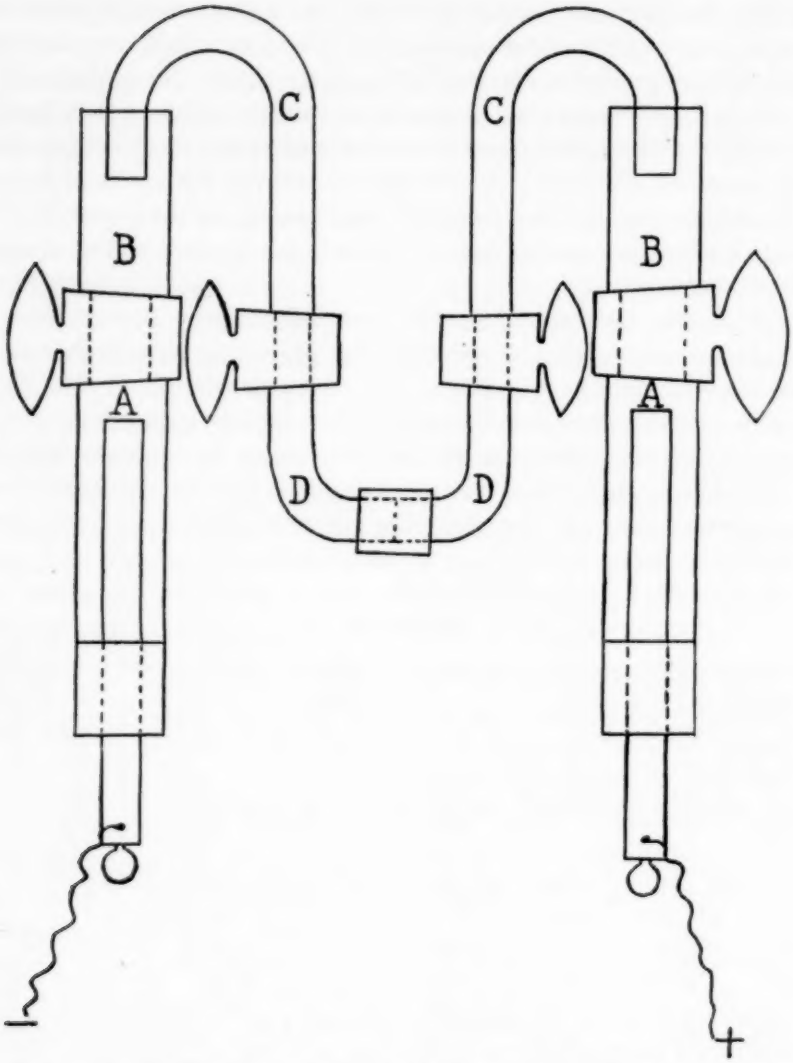

FIG. 1. Apparatus for cataphoresis of red blood cells. A, $ZnSO_4$; B, buffer; C, saccharose; D, cell suspension.

small stop-cocks with solution, the two halves of the middle portion can be filled separately with the test suspension and then pressed together, the short length of rubber tubing being left attached to one

of the halves. The ends of the side arms are then hooked into the electrode tubes containing buffer solution, and the cocks carefully opened. The fluid in the middle portion is brought to the same level on each side by adding a small amount, one or two drops, of buffer solution to the buffer chamber on one side or the other. With this type of apparatus the fall in electrical potential across the U-tube is relatively great, and the change in hydrogen ion concentration in the test fluid during the passage of current is reduced to a minimum. The determinations were made at 19 ± 1°C. without a thermostat in a room of even temperature. The current used was the Edison direct street current of 220 volts, giving a drop of almost exactly 7 volts per cm. in the U-tube.

Fresh defibrinated sheep blood was washed with two or more changes of saline solution and then freed from electrolyte by washing with four changes of ten volumes each of isotonic saccharose solution (M/4), and finally made up to 10 per cent concentration of cells in saccharose. It was found that the reaction of the saccharose solution should not be more alkaline than pH 6.5 as at more alkaline reactions the viscidity of the sedimented cell mass is so great that it is reemulsified only with difficulty and with the development of considerable hemolysis.

To determine the direction and rate of movement of red cells at varying hydrogen ion concentrations, a titration was first carried out to determine the H concentration resulting from the addition of graded amounts of HCl to the cell suspension at hand. 5 cc. of cells were introduced into twelve or more tubes, in two series. To the first series of tubes were then added 5 cc. of saccharose solution and increasing amounts of M/10 or M/100 HCl, the latter dilution made up in saccharose. Between manipulations the tubes and the stock flask were kept tightly stoppered to exclude atmospheric CO_2 as far as possible. After gentle agitation the tubes were allowed to stand at room temperature for 10 to 15 minutes, then centrifugated and the supernatant fluid was drawn off. Of this, one portion of 5 cc. with indicator added was compared with a standard series for the colorimetric determination of hydrogen ion concentration, the remaining portion serving as a color screen for the standard tube. The value thus obtained represents the H concentration with which the cells were in equilibrium.

The test emulsion for cataphoresis was prepared by adding the proper amount of acid to secure the desired H concentration to 5 cc. of saccharose solution; this was then poured gently into one of the second series of tubes of cells, and the mixture pipetted immediately into the U-tube. In this way it was possible even at reactions near the isoelectric point to observe the movement of cells before agglutination was complete and in many cases before macroscopic agglutination had appeared. For each determination 10 cc. of buffer solution and 10 cc. of saccharose solution, of the H concentration of the tube of cells to be examined, were prepared. If the apparatus was carefully manipulated the boundary of contact between the cells and the fluid in the side arms remained sharp, and the distance of displacement of the boundary during passage of current could be measured quite accurately with a pair of dividers. When agglutination appeared, a more dilute suspension remained, the movement of which could be determined likewise.

In addition to the determinations made in the absence, as far as possible, of electrolyte, measurements were made of the migration of cells in the presence of sodium chloride, sodium acetate, and sodium phosphate, using a dilution of 1 part of isotonic solution of these salts to 4 parts of saccharose solution. The acetate and phosphate were used in the form of buffer mixtures.

Sensitized cells were prepared by adding to a 10 per cent suspension of cells in saccharose approximately 50 hemolytic units of a high titer immune rabbit or hare serum. The reaction of the mixture was about pH 6.0. After incubation for 2 hours at 37°C. the cells were either used at once or were placed in the refrigerator over night. Before use the cells were sedimented twice and again made up to 10 per cent concentration. The amount of electrolyte in the immune serum was insufficient to cause agglutination. Kosakai[11] has shown that sensitizer can be dissociated from red cells by extraction in salt-free solutions of various sugars; we have been able to confirm this, but the dissociation is not complete and the cells used in our determinations were still combined with sensitizer, as shown by prompt hemolysis on the addition of complement and by agglutination when added to saline

[11] Kosakai, M., *J. Immunol.*, 1918, iii, 109.

solution of the usual strength. The titrations for H concentrations of the sensitized cells were carried out according to the method already described.

The determinations for normal and sensitized cells have been recorded in the form of curves (Figs. 2 and 3) in which the millimeters of movement during 10 minutes time are plotted as ordinates against the pH values as abscissæ. From these curves it appears that the

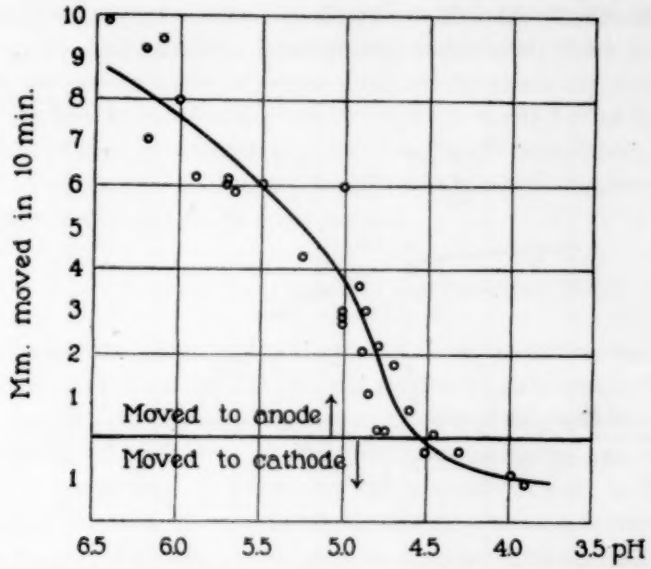

FIG. 2. Curve showing the movement in mm. during 10 minutes of normal red blood cells at varying hydrogen ion concentrations. The points above the curve between pH 4.0 and 5.0 represent determinations on different specimens of blood from those recorded in the points below the curve, so that the difference may represent actual variations in individual specimens.

direction and rate of movement in the electric field of both normal and sensitized red blood cells is a function of the hydrogen ion concentration. At concentrations less than pH 4.6 the charge carried is negative and increases in amount with the alkalinity; pH 4.6 represents the isoelectric point; at concentrations greater than pH 4.6 the charge carried is positive and increases in amount with the acidity.

A comparison of the two curves shows that on the alkaline side of the isoelectric point the charge of normal cells is greater and increases

more rapidly with increasing alkalinity than the charge of sensitized cells. The slower movement cannot be explained by the formation of agglutinated masses, since at the upper end of the curve at least no agglutination appeared even after 12 hours standing in the U-tube. On the acid side of the isoelectric point the measurements are not numerous and probably not accurate enough for a comparison.

The optimum H concentration for agglutination was determined by titrations exactly like those described above. Varying amounts of cells were used; the sharpest results were obtained by adding 1 or 2

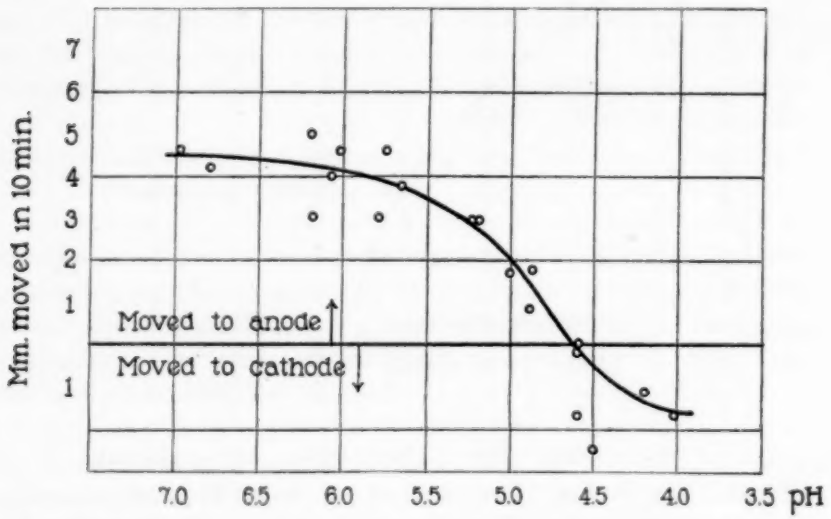

FIG. 3. Curve showing the movement in mm. during 10 minutes of sensitized red blood cells.

cc. of 10 per cent cells to 10 cc. of saccharose solution containing increasing amounts of HCl. At the optimum concentration agglutination is almost instantaneous; at greater and less acidities agglutination appears more slowly, but was practically completed within 30 minutes. The agglutination arranges itself asymmetrically on either side of the optimal point; with normal cells it never was observed at reactions more alkaline than pH 5.6, and seldom beyond pH 5.1. This variation is probably connected with variations in the amount of electrolyte present, and will be considered later. On the acid side

of the optimal point, however, agglutination appeared at all concentrations. As a result of over thirty series of determinations the optimum for agglutination of normal red cells may be given as pH 4.75. In the great majority of experiments the optimum corresponded quite sharply with this figure.

With sensitized cells, a greater variation was noted in individual experiments, due possibly to varying degrees of sensitization, but when the cells were still heavily sensitized after the final washing, the optimum for agglutination occurred regularly near pH 5.3. The optimum for precipitation of serum globulin, in which fraction of the serum the immune bodies are carried, is given as pH 5.2 by Rona and Michaelis,[12] and the isoelectric point of typhoid immune body[10] as pH 5.4 so that the flocculation of sensitized cells seems to be connected with that of the specific immune serum.

The Chemical Significance of the Isoelectric Point.

From the data given by the titrations above described, and carried out for the determination either of the isoelectric point or the optimum for agglutination, one may construct a curve giving the amounts of HCl added, as ordinates, on the pH values attained, as abscissæ. A similar curve may be drawn for the addition of acid to 10 cc. of saccharose solution alone. From these two curves a third may be drawn (Fig. 4) with pH values again as abscissæ, and as ordinates the differences between the ordinates of the first two curves, or the amounts of HCl required to bring a suspension of cells to a given H concentration in excess of those necessary in the case of the saccharose solution alone. There is evident a sharp inflection in this curve at pH 4.7. This point is so close to the value found for the isoelectric point that we may speak of it as the same. The protein of the cells exists then on the acid side of the isoelectric point as a different chemical substance from that occurring on the alkaline side. Since a true "puffer" action is manifest, it appears that the protein combines with hydrogen ion. An adsorption of hydrogen ion can hardly be thought of here as anything but a chemical combination.

[12] Rona, P., and Michaelis, L., *Biochem. Z.*, 1910, xxviii, 193.

The observation by Joos[13] that the salt necessary for the agglutination of sensitized bacteria combined chemically with the bacteria, since it was not to be detected in the supernatant fluid after agglutination, although not confirmed by later investigators, led me to investigate the removal of salt after acid agglutination. With cells washed as free as possible from electrolyte, the amount of chloride present was very small and consisted chiefly in that added as HCl. A test for chlorine ion was made with $AgNO_3$ on the supernatant fluid from the cells, and it was found that at pH 4.8 no chloride was present, and

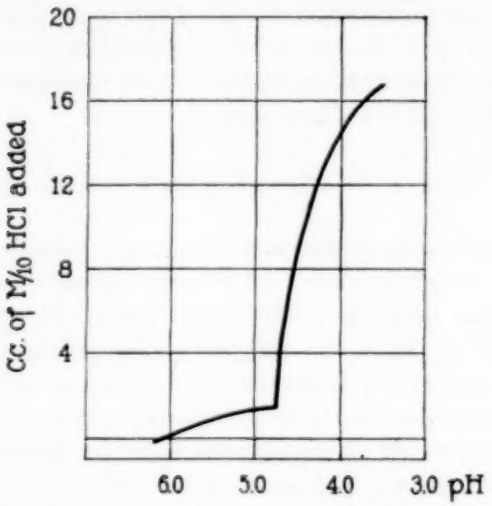

FIG. 4. Curve showing the combination of red blood cells with hydrogen ion. 5 cc. of 10 per cent normal cells. The same curve is given with sensitized cells.

only the slightest trace in the tubes to which considerable amounts of HCl had been added, while on the alkaline side of pH 4.8 a distinct turbidity appeared.

This experiment was repeated many times, always with the same result. The conditions were determined more accurately by volumetric analysis of the fluid from tubes of cells to which small amounts of M/10 NaCl were added along with M/10 HCl, so that each tube contained exactly the same amount of chlorine ion. The results are

[13] Joos, A., *Z. Hyg.*, 1901, xxxvi, 422.

given in the form of curves, Fig. 5 being that for normal cells and Fig. 6 that for sensitized cells. The curves give as ordinates the gram-ions $\times 10^{-5}$ of Cl lost by the supernatant fluid, or gained by the cells. It will be seen that the chlorine ion combines with both normal and sensitized cells in much larger amount on the acid side of pH 4.7 than

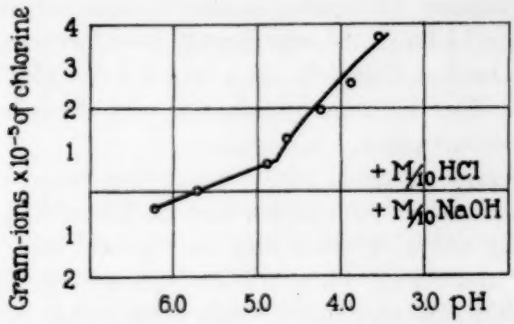

FIG. 5. Curve showing the combination of normal red blood cells with chlorine ion. 5 cc. of 10 per cent cells.

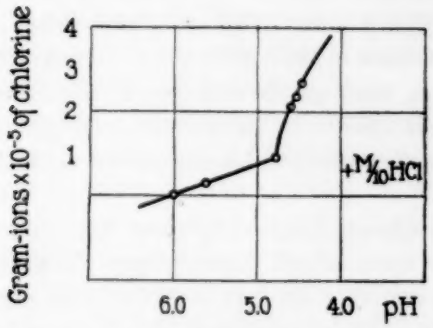

FIG. 6. Curve showing the combination of sensitized cells with chlorine ion. 5 cc. of 13 per cent cells.

on the alkaline side. On adding NaOH, Cl is actually given up by the cells between pH 5.7 and 6.2. This effect cannot be due merely to an increase in permeability of the cells, permitting the escape of chlorine ion, for in that case the amount of chlorine ion actually found should have been greatest at pH 3.5, where a trace of hemolysis appeared.

A similar determination was made on the combination with a cation at different pH values. Cells were suspended in isotonic $BaCl_2$, then washed, and again suspended in saccharose solution which had been brought to pH 6.0 with $Ba(OH)_2$ instead of NaOH. The amount of Ba present was therefore small, and could only be estimated from the turbidity of the fluid on adding Na_2SO_4. Many of the experiments showed no significant differences; in several, however, it was found that the amount of Ba in the supernatant fluid increased sharply at pH 4.7 and showed a further increase to pH 4.0. This must mean that the Ba was liberated from combination with the cells on the acid side of the isoelectric point.

Exactly the same chemical behavior has been found by Loeb[1] for gelatin, which combines with cation only on the alkaline side of its isoelectric point and with anion only on the acid side. This Loeb has shown by a series of volumetric analyses for Ag, Br, and CNS ions of the gelatin-ion compound. This work was unfamiliar to the present author at the time the observations detailed above were carried out. It is significant of the general importance of this relation that the same chemical behavior is manifested both by solutions of gelatin and by living cells. The old observation of Hamburger[14] that under the influence of HCl, H_2SO_4, or CO_2 red cells take up chlorine from the serum, while on the addition of alkali they give chlorine up to the serum, is thus to be interpreted, as suggested by Loeb, by the difference in chemical combining power on either side of the isoelectric point.

The conclusion appears justified by these facts that the character of the charge carried by a red cell depends upon the nature of its chemical combination; and that the amount of charge depends upon the amount of protein in ionic combination. This conclusion was facilitated by the observations of Loeb, already referred to.

The curve for velocities of normal cells (Fig. 2) on the alkaline side of the isoelectric point closely parallels the curve given by Loeb for swelling and conductivity of Na gelatin. Korányi and Bence[15] have shown that between saturation of defibrinated blood with CO_2 on the

[14] Hamburger, H. T., Osmotischer Druck und Ionenlehre, Wiesbaden, 1902, i.
[15] Korányi, A., and Bence, J., *Arch. ges. Physiol.*, 1905, cx, 513.

one hand and removal of the CO_2 by a stream of oxygen, on the other, the values for viscosity of the blood and refractive index of the serum pass through the minima. From the data already given, it appears that we must interpret these minima as corresponding with the isoelectric points of the serum and the cells, although in the lack of definite values for the reaction at which these minima occurred, this conclusion must be considered probable rather than proved. Indeed this conclusion was foreshadowed by Korányi and Bence in their interpretation of Höber's observations[4] on the migration of red cells.

DISCUSSION.

The effect of the addition of sodium chloride, sodium phosphate, or sodium acetate upon the velocity of cells, on the alkaline side of the isoelectric point at least, is a minor one. As compared with the values in the absence of electrolyte the velocities are somewhat greater in the presence of NaCl, and somewhat less in the presence of the other salts. These variations are in all probability due to alterations in the viscosity of the medium, which was not corrected for in the measurements given in the curves. This close correspondence of values is what we should expect if the cells exist at these reactions only as sodium proteinate, in these experiments.

When normal cells are washed in four or more changes of saccharose solution, a very slight absorption of CO_2 from the atmosphere is sufficient to raise the H concentration to pH 4.75, at which the cells agglutinate almost instantaneously. In this condition they exist most nearly in the pure state, uncombined with acid or base.

The addition of NaCl on the other hand lessens their susceptibility to change in reaction. When this salt or $CaCl_2$ is added the pH reached is less acid for a given amount of HCl than if the cells are suspended in saccharose solution alone. It is this decrease in H concentration in the presence of salt, without doubt, which carried the reaction in Höber's experiments from the acid to the alkaline side of the isoelectric point and thereby prevented the saturation of the solution with CO_2 from bringing about a positive charge on the cells. The pH value for the isoelectric point is not altered, but it is attained only on the addition of larger amounts of HCl. The curve for pH

values shows the same inflection at pH 4.7 as Fig. 4, and is therefore omitted.

This effect is very difficult to analyze. It appears to be a chemical one, and may involve the formation of a small amount of protein chloride on the alkaline side of the isoelectric point, due to the excess of chlorine ion, but since the effect is noted on both sides of the isoelectric point, any explanation of it in the light of the facts presented here would be largely speculative.

The agglutinability of sensitized cells in the presence of an electrolyte as compared with the stability of normal cells is not an absolute difference, even when the electrolyte is NaCl. Specific agglutination is known to occur over a wide range of pH values, as shown by Krumwiede and Pratt[16] among others; that it is not independent of the hydrogen ion concentration, however, is indicated as shown above by the existence of an optimum, at which it is practically independent of the presence of neutral salt. On either side of this range as common experience shows it is accelerated by the presence of neutral salt. Similarly normal cells which may not agglutinate in salt-free media at pH 4.9 have been found in our titrations to agglutinate readily at pH 5.5 in the presence of NaCl. At less acidities, while flocculation may not occur, we have found the speed of settling to be affected in a similar way by the hydrogen ion concentration.

It is apparent then when we compare the influence of the hydrogen ion concentration upon the electrical charge and chemical behavior of red cells with the influence upon agglutination that we have to deal not with one phenomenon, but with two, which are, however, closely related.

CONCLUSIONS.

1. The movement of normal and sensitized red blood cells in the electric field is a function of the hydrogen ion concentration. The isoelectric point, at which no movement occurs, corresponds with pH 4.6.

2. On the alkaline side of the isoelectric point the charge carried is negative and increases with the alkalinity. On the acid side the charge is positive and increases with the acidity.

[16] Krumwiede, C., Jr., and Pratt, J., *Z. Immunitätsforsch., Orig.*, 1912, xvi, 517.

3. On the alkaline side at least the charge carried by sensitized cells is smaller and increases less rapidly with the alkalinity than the charge of normal cells.

4. Both normal and sensitized cells combine chemically with inorganic ions, and the isoelectric point is a turning point for this chemical behavior. On the acid side the cells combine with the hydrogen and chlorine ions, and in much larger amount than on the alkaline side; on the alkaline side the cells combine with a cation (Ba), and in larger amount than on the acid side. This behavior corresponds with that found by Loeb for gelatin.

5. The optimum for agglutination of normal cells is at pH 4.75, so that at this point the cells exist most nearly pure, or least combined with anion and cation.

6. The optimum for agglutination of sensitized cells is at pH 5.3. This point is probably connected with the optimum for flocculation of the immune serum body.

The author wishes to express his indebtedness to Dr. K. George Falk, of the Harriman Research Laboratory, the Roosevelt Hospital, New York, for criticism and suggestions.

THE PRODUCTION OF VOLATILE FATTY ACIDS BY BACTERIA OF THE DYSENTERY GROUP.

By HARPER F. ZOLLER and W. MANSFIELD CLARK.

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(Received for publication, October 11, 1920.)

INTRODUCTION.

In the course of an investigation of bacteria of the dysentery group we had occasion to determine the volatile fatty acids produced by these bacteria under different conditions. We believe the results to have considerable significance but their interpretation had best wait upon the development of more fundamental knowledge of anaerobiosis than we now possess. We therefore desire to place the following data on record without comment.

Organisms Studied—The cultures were secured from several investigators and institutions and represented the usual types together with some atypical organisms. A laboratory fire destroyed the history of each culture, but they have been separated into Shiga and non-Shiga types by mannite fermentation tests through the cooperation of Dr. J. M. Sherman of this laboratory. Fermentation data were at hand which would permit the differentiation of the non-Shiga cultures into Flexner, Strong, and other strains; but this was not done, since the results of the volatile acid work are in such close agreement that, as conducted, they have no important bearing upon the classification of the dysentery bacteria. The results from one each of typhoid (Ty Round) and paratyphoid (P Ty 16) cultures are included because of the relation existing between the dysentery and these groups of organisms. Following the laboratory number will be found the strains studied: D_1, Flexner; D_2, Shiga; D_3, Shiga; D_4, Flexner; D_5, Shiga; D_6, non-Shiga; D_8, non-Shiga; D_{10}, non-Shiga; D_{11}, non-Shiga; D_{13}, non-Shiga; D_{17}, non-Shiga; D_{23}, non-Shiga; D_{24}, Shiga; D_{33}, non-Shiga; D_{37}, Shiga.

Methods.

Media.—The medium for the growth of the organisms consisted of 1.0 per cent of "Difco" peptone, and 0.5 per cent of $Na_2HPO_4 \cdot 2H_2O$, with or without 1.0 per cent of Merck's "highest purity" dextrose. The reaction of this medium after sterilization always lay between pH 7.1 and 7.2. The hydrogen ion concentrations of the media and cultures were determined colorimetrically.

Cultures.—The organisms were inoculated into sterile 10 cc. portions of the media and incubated at 37°C. for 18 hours. These virile cultures were then poured into 990 cc. of the sterile media contained in 2 liter Florence flasks and incubated at 37°C. for the period of time indicated. The pouring was conducted under conditions which reduced the chance for contamination by this method. No control plates were run to determine this factor, since it was held that the bulky inoculation was sufficient to take care of any slight contamination should such occur. The regularity in the content of volatile fatty acids leads us to believe that there was no contamination. It was found that the maximum production of total volatile acids in sugar medium under these conditions occurred shortly before the first 12 hour period and the total quantity remained quite stationary during the following 60 hours (Fig. 1). Accordingly, the cultures were allowed to become 48 hours old merely as an arbitrary period. The cultures in non-sugar media were allowed a longer period before examining for volatile acids, because volatile bases were investigated in the same cultures. It is barely possible that the values for the volatile acids obtained in the case of these cultures grown for a longer period of time are somewhat too low because of the chance of volatile acid utilization.

Anaerobic Cultures.—"Anaerobic" conditions were obtained by evacuation of the inoculated flasks with a mercury-vapor pump (a single Kraus modeled pump) in series with a motor-driven Geryke vacuum pump. Gum stoppers, bearing a thick-walled tube drawn out to a narrow neck to permit sealing with a fine tipped flame, were cemented while hot with a heavy rubber-rosin cement into the necks of the flasks. These stoppers had been previously sterilized, and the glass tubes contained a plug of cotton to prevent contamination of the

flasks while evacuating. After pumping out the bulk of the air with the Geryke pump till the Plücker tube discharge showed a declining spark, the mercury-vapor pump was started and it was allowed to pump for 20 minutes more or until the Plücker tube discharge showed a continued decline in brilliancy. The flasks were sealed off while the pumps were in operation.

Separation of the Volatile Acids from the Cultures.—10 per cent sulfuric acid was added to the cultures until a drop of thymol blue showed a distinct red; this represented a pH of 1.0 or less. Steam formed by boiling distilled water in the presence of barium hydroxide was led into the flasks holding the cultures. The flasks in turn were connected to vertical condensers through which ice-cooled water circulated. The condensers were arranged vertically to facilitate the delivery of the condensed fatty acids. A small piece of paraffin was placed in the cultures to overcome frothing during distillation. The volume of liquid in the culture flasks was held constant by means of a screened flame. Fractions of 500 cc. each were caught and titrated with 0.1 N NaOH to phenolphthalein. The distillation was continued until the last fraction required no more than 2 cc. of 0.1 N NaOH. Usually ten to fourteen fractions were collected. These were evaporated *in toto* and made up to a definite volume.

Distillation was conducted upon a like volume of sterile medium to determine the factor for carbon dioxide, etc.

Quantitative Analysis of the Volatile Fatty Acids.—The modified Duclaux method published by Gillespie and Walters[1] was followed in the main in the determinations. It was found advisable to determine new constants for formic, acetic, propionic, and normal butyric acids with the distillation apparatus employed. The still was of Jena glass throughout and contained a small, closed end, inverted tube in the solution to insure against excessive spattering and to produce a steady flow of vapor. The constants found did not depart radically from those obtained by Gillespie and Walters. The still flask was heated on the sides and bottom with electrically heated coils.

An aliquot of the total volatile acid concentrate was employed which contained from 60 to 80 cc. of 0.1 N volatile acids.

[1] Gillespie, L. J., and Walters, E. H., *J. Am. Chem. Soc.*, 1917, xxxix, 2027.

The mode of treating the results depended somewhat upon the number of volatile acids found to be present in the cultures under examination. For only two acids the algebraic method[1] (page 2036) was used, although the graphical method (page 2040) was found to be somewhat more rapid. When three acids were found to be present the graphical method mentioned by Gillespie and Walters (page 2054) was followed. This latter method involved much less calculation than the algebraic and was equally accurate.

As a check on the modified Duclaux method, the formic acid was determined in several cultures by the mercuric chloride method of Franzen and Egger.[2] The two methods gave values for this acid harmonizing within 2 per cent of the whole. It is assumed that the values found for the other acids are as accurate.

In the cultures containing butyric acid its presence was further established by preparing the quinine salt as outlined by Phelps and Palmer[3] and determining its melting point. The distillation values accurately establish the identities of the acids, provided the manipulations are carefully conducted and the constants are determined with purified acids.

Sugar determinations were conducted upon the media and several cultures to obtain data regarding the utilization of the sugar and the amount of acid formed therefrom. From 30 to 40 per cent of the sugar was utilized by the bacteria in cultures 2 and 14 days old. It was at first feared that, because the total quantity of volatile acids produced by each organism in the same length of time reached about the same value, not enough glucose was present. It is evident, however, if we view Fig. 1 in the light of this sugar utilization, that the organisms growing in sugar media reached their limiting zones of volatile fatty acid production in the presence of sugar. This may have an important bearing on the growth curve of bacteria. This large yield of formic acid by these bacteria from glucose suggests a possible commercial problem.

Table I contains a summary of the determinations under the four sets of conditions. Fig. 1 pictures the results of the progressive series

[2] Franzen, H., and Egger, F., *J. prakt. Chem.*, 1911, lxxxiii, 323.

[3] Phelps, I. K., and Palmer, H. E., *J. Biol. Chem.*, 1917, xxix, 199.

studies and is included to furnish justification for choosing the 48 hour cultures for the main study.

TABLE I.

Volatile Fatty Acid Production of Dysentery Bacteria Under Different Conditions of Growth.

Organism.	Age of culture.	pH of culture.	Sugar present.	State.	Acid produced.			
					Formic. N/10	Acetic.	Propionic.	Butyric.
	days		*per cent*		*cc.*	*cc.*	*cc.*	*cc.*
D_1	2	4.9	1	Aerobic.	128.7	88.2	None.	None.
D_2	2	5.0	1	"	122.3	82.9	"	"
D_3	2	5.0	1	"	132.9	88.5	"	"
D_4	2	5.2	1	"	172.2	83.8	"	"
D_5	2	5.0	1	"	121.5	92.5	"	"
D_6	2	4.8	1	"	127.2	78.5	"	"
D_8	2	4.9	1	"	149.3	92.3	"	"
D_{10}	2	5.0	1	"	143.5	89.5	"	"
D_{11}	2	4.8	1	"	145.0	102.1	"	"
D_{13}	2	4.9	1	"	124.3	76.5	"	"
D_{17}	2	5.2	1	"	145.2	66.0	"	"
D_{23}	2	5.4	1	"	86.5	60.8	"	"
D_{24}	2	5.2	1	"	112.3	83.0	"	"
D_{33}	2	5.2	1	"	169.5	88.0	"	"
D_{37}	2	4.8	1	"	118.5	73.3	"	"
P Ty 16	2	5.1	1	"	77.3	80.2	"	"
Ty Round	2	5.1	1	"	150.5	78.6	"	"
D_1	10	7.5	None.	"	None.	33.2	12.8	"
D_2	10	7.6	"	"	"	35.2	13.3	"
D_4	10	7.5	"	"	"	27.9	11.1	"
D_{37}	12	7.4	"	"	"	31.5	11.8	"
D_1	12	6.2	"	Anaerobic.	31.2	27.1	None.	5.5
D_2	12	6.4	"	"	15.3	47.4	"	7.1
D_4	12	6.8	"	"	31.2	32.2	"	7.4
D_{37}	12	6.8	"	"	Determination lost.			
D_1	12	5.2	1	"	90.1	77.0	None.	None.
D_2*	14	5.2	1	"	87.5	56.5	"	"
D_4	12	5.5	1	"	116.4	78.2	"	"
D_{37}	12	5.4	1	"	94.8	68.5	"	"

* Growth less heavy to the eye.

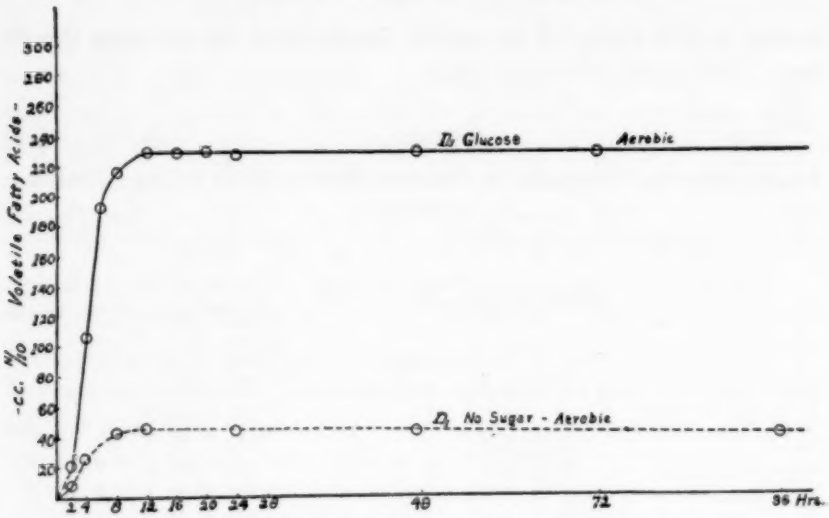

FIG. 1. Volatile fatty acid production curves of D_1 grown in 1,000 cc. of medium, and under the conditions indicated.

CONCLUSIONS.

These studies show:

1. A close agreement exists among all the organisms studied in the total quantity of volatile fatty acids produced and in the ratio of formic to acetic, under aerobic conditions, and in the presence of 1 per cent of glucose.

2. When grown upon peptone alone, with free access of air to the cultures, volatile fatty acids are produced in appreciable quantities, although the reaction of the solution has gone more alkaline as shown by colorimetric pH tests. Formic acid is not found, but in its place we obtain propionic acid.

3. Upon exhaustion of air from the non-sugar medium the bacteria again produce formic acid, and in addition some butyric. This is true for both Shiga and non-Shiga cultures. The reaction is distinctly more acid.

4. The presence of glucose in the medium from which the air has been pumped furnishes a condition which provokes about the same type and degree of fermentation that operates in the glucose medium bathed in air at atmospheric pressure.

5. The enormous quantity of formic acid produced by these bacteria may play a significant part in the digestive disturbances and toxic symptoms accompanying their infection of the human intestinal tract.

THE RESPONSE OF POPILLIA JAPONICA TO LIGHT AND THE WEBER-FECHNER LAW.

By A. R. MOORE and W. H. COLE.*

(From the Riverton Entomological Laboratory, Riverton, N. J.)

(Received for publication, October 26, 1920.)

The Japanese beetle, *Popillia japonica*, is a recent importation from Japan and is found in a limited area in central New Jersey.

During July and the first half of August the beetles may be seen collected in great numbers at the tops of trees, bushes, and weeds. It seems to be immaterial whether the plant is a linden tree 50 feet high or a smartweed plant a foot high—the beetles always occupy the uppermost foliage. This is due to the fact that the direction of movement in these beetles is the additive resultant of two tropistic responses; namely, positive phototropism and negative geotropism. These tropisms determine the head-tail orientation of the body.

Both in the field and in the laboratory a certain degree of heat and of light is necessary for the active movements of *Popillia*. Thus, below 23°C. the beetles are generally inactive; 38–39°C. is optimum for their activity, while above 40°C. injurious effects are apparent. At 45°C. activity ceases quickly and permanently. Under the ruby light and in the dark most of the beetles become quiet and show no response to gravity. Occasional individuals which do move show a retarded response. All are roused to activity by illumination from any direction, while a great increase in the strength of the light causes marked acceleration of movement and flying.

In addition to this kinetic action of light there is also a directive effect, which may be demonstrated by illuminating the beetles first from above and then from below and timing their response in each case. Without exception they ascend the wire gauze of their cage more rapidly in the first instance, although in the latter case the

* The apparatus for this work was furnished by Rutgers College, New Brunswick, N. J.

animals eventually reach the top, thus proving the geotropic response to be the major factor in orientation. Table I shows the retarding effect of illumination from below. Incidentally these figures well illustrate the constancy with which any given lot of animals responds. It is apparent from this result that while light exercises a directive action, the directive effect of gravity is greater than that of light of the strongest intensity used.

Since the geotropic response is shown by the beetles only when illuminated, it therefore follows that their movement in a lighted field is the result of three factors; *viz.*, negative geotropism, photo-

TABLE I.

*Light Intensity 3,276 Candle Meters.**

Light above.	Light below.
Reaction time.	Reaction time.
sec.	*sec.*
10	15
11	16
9	17
11	17
11	15
Mean.....10.4	16

* With the light above, gravity and light act together to produce a response in 10.4 seconds. When the light is below, the directive action of the light acts against the effect of gravity and thus delays the response to 16 seconds.

kinesis, and positive phototropism. The first is constant, the other two factors are functions of the intensity of the illumination. In the case in which the light is from above, it follows that changes in the rate of movement of the beetles depend on variations in the degree of photokinesis and of phototropism. These factors in turn depend upon the intensity of the illumination. Hence we may express the relation of the rate of response to the intensity of the illumination by saying that the rate of response is a function of the light intensity.

In the present study, the determinations of reaction time were made as follows. A cage of wire gauze 12.5 cm. high was constructed, having a movable top of the same material. During a series of

experiments this cage containing twelve female beetles was kept in a thermostat made of glass at a temperature of approximately 38.5°C. with extreme variations for short intervals on two or three occasions during the season to 37.5 and 39.5°C. Five intensities of illumination were secured by the use of Mazda glowers of different powers. During the experiment the glower was held by a clamp 25 cm. from the top of the cage. The latter was protected from the heat of the lamp by a water screen. In making the observations one observer manipulated the cage by inverting it for each start and then noted the instant that six beetles (50 per cent of all) reached the top of the cage. The start and stop were signalled to the second observer who kept time with the stop-watch and recorded the time intervals to the nearest

TABLE II.*

I	$\log_e I$	Reaction time.	$R = \frac{100}{\text{Reaction time}}$	$R = \frac{\log_e I + K}{k}$
			Observed.	Calculated.
		sec.		
85	4.44	15.50	6.45	6.56
234	5.45	13.14	7.61	7.49
608	6.40	11.60	8.62	8.36
1,600	7.37	10.86	9.21	9.25
3,276	8.08	10.22	9.78	9.90

* I = the intensity of the light in candle meters, R = rate at which the organ isms respond, $k = 1.09$, and $K = 2.71$.

second. Five observations were made at each intensity with each lot of twelve animals. Seven such series were run. Each point is therefore the result of thirty-five observations. The mean of the values obtained for each intensity is given under the heading "Reaction time" in Table II. The rate is equal to $\frac{100}{\text{Reaction time in seconds}}$.

We have assumed that the rate of movement is an objective measure of the effect of light on the organism, *i.e.* sensation, and now proceed to consider our experimental results in the light of the Weber-Fechner concept. According to Weber's law, the least noticeable difference of a stimulus is proportional to the magnitude of the pre-existing stimulus. Fechner[1] has expressed this relation in the form

[1] Fechner, G. T., Elemente der Psychophysik, Leipsic, 1860, i, 211.

of a differential equation according to which $dS = c.\frac{dI}{I}$, in which S = the magnitude of sensation, I is the intensity of the exciting cause, and c is the constant of the series. Integrating, this equation becomes $S = c.\log_e I + K$. This means that the sensation is proportional

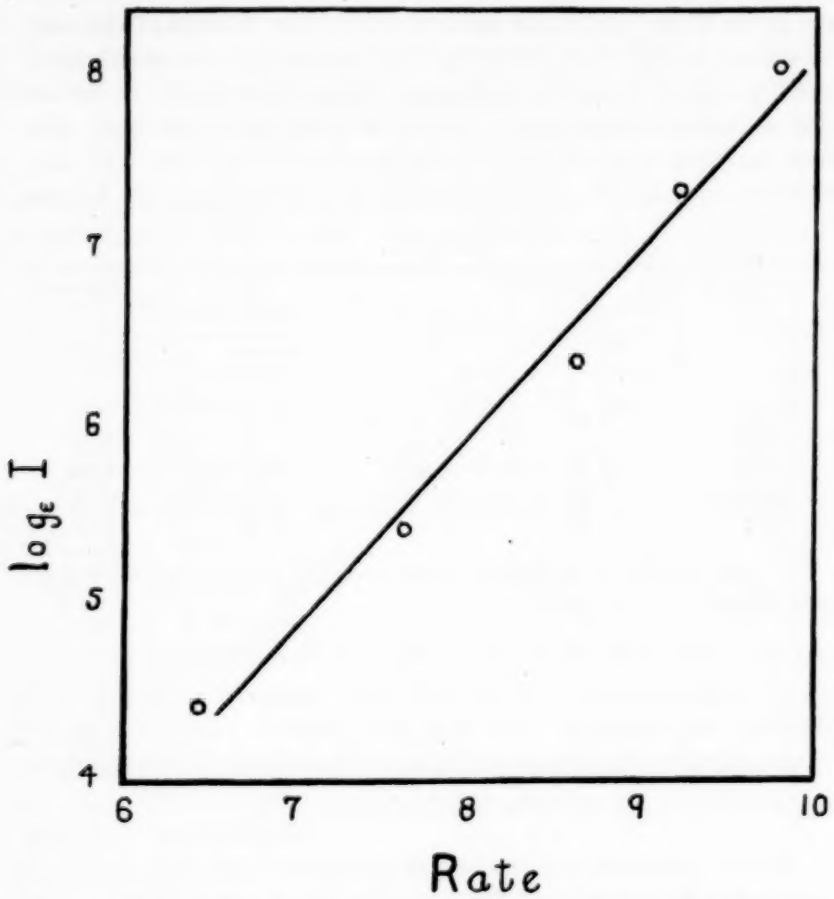

FIG. 1. The small circles show the experimental points in which Rate = $\frac{100}{\text{Reaction time in seconds}}$; the line is drawn through the points calculated for the rate by means of the equation, Rate = $\frac{\log_e I + K}{k}$.

to the logarithm of the intensity of the stimulus. Putting R, the rate of response, for S, transposing and putting k for $\frac{1}{c}$, the integrated equation of Fechner becomes $\log_e I = kR - K$.

Substituting the experimental values of $\log_e I$ and of R in this equation and solving by the method of least squares for k and K we obtain $k = 1.09$ and $K = 2.71$. These quantities when put into the equation for each value of $\log_e I$ in turn yield a series of calculated values for R, as shown in the last column of Table II.

When the rates are plotted as abscissæ against the values of $\log_e I$ as ordinates the result is a straight line (Fig. 1), showing at a glance that the rate of response of the organism to light is proportional to the logarithm of the intensity of the illumination. This demonstrates that the Japanese beetle responds to light in accordance with the Weber-Fechner law.

SUMMARY.

Light and a temperature above 23°C. are necessary for the activity of *Popillia*.

The effect of light as indicated by the rate of locomotor response is related to light intensity according to Fechner's expression of Weber's law.

COMPARATIVE STUDIES ON RESPIRATION.

XIV. Antagonistic Action of Lanthanum as Related to Respiration.

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(From the Laboratory of Plant Physiology, Harvard University, Cambridge.)

(Received for publication, July 8, 1920.)

In previous papers[1,2] the writer has discussed the relation between respiration and antagonism, including antagonism between monovalent cations and bivalent cations. The present paper extends these studies to trivalent cations.

The experiments were made upon *Bacillus subtilis*. The method of experimentation has been fully described in previous papers.

The results are shown in the figures. Fig. 1 shows the manner in which the rate of respiration changes under the influence of $La(NO_3)_3$ in concentrations of 0.000006, 0.000025, 0.00005, 0.05, and 1.0 M. During the first 10 minutes the bacteria are under normal conditions and the curve (broken line) is horizontal. After this (at the point marked 0 on the abscissa) the salt is added. The addition of sufficient $La(NO_3)_3$ to make the concentration 0.000006 M produces an increase in the rate which remains constant during the period of experimentation. When the concentration is 0.000025 M the rate is normal, while in higher concentrations there is a decrease in rate which amounts almost to a cessation of respiration at 1 M. These curves are selected from a number of similar typical curves and each represents one experiment.

Fig. 2 shows the effect of various concentrations of $La(NO_3)_3$ upon the rate of respiration. The rate indicated is that produced after the bacteria had been in contact with the salt for 1 hour. There is an increase in the rate of production of CO_2 at 0.000006 M and a decrease in the rate at concentrations higher than 0.000025 M. As the effect

[1] Brooks, M. M., *J. Gen. Physiol.*, 1919–20, ii, 5.

[2] Brooks, M. M., *J. Gen. Physiol.*, 1919–20, ii, 331.

of the lower concentrations cannot be clearly shown in the figure the results are given in Table I.

Fig. 3 shows antagonism. In this figure the abscissæ represent the number of cc. of each component in the mixture; since the molecular concentrations are the same for all the components the abscissæ

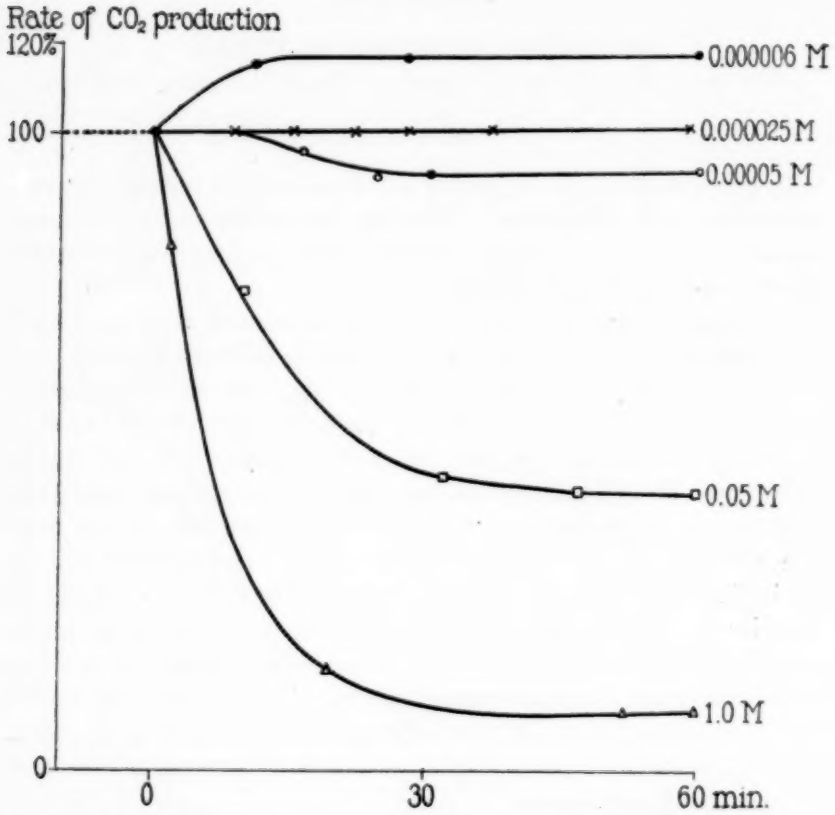

FIG. 1. Curves showing the rate of respiration of *Bacillus subtilis* (expressed as per cent of the normal) in 0.000006, 0.000025, 0.00005, 0.05, and 1.0 M $La(NO_3)_3$. The zero point on the abscissa denotes the beginning of exposure to the salt solution; previous to this the bacteria were in 0.75 per cent solution of dextrose in distilled water. The normal rate (which is taken as 100 per cent) represents a change in pH value from 7.78 to 7.60 in a number of seconds depending upon the amount of bacterial suspension used, usually 30 seconds. Each experiment is represented by a single curve.

represent molecular proportions. The ordinate in the center represents the rate of respiration in a mixture of 50 cc. of each component; the ordinate at the extreme left represents the rate in a pure solution of one component; the ordinate at the extreme right, the rate in a

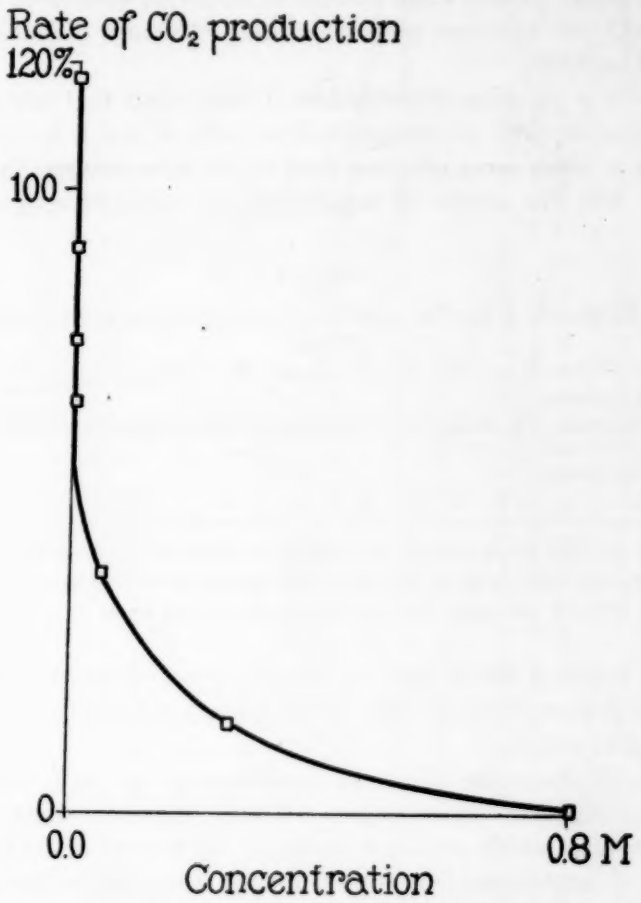

FIG. 2. Curve showing the rate of respiration of *Bacillus subtilis* (expressed as per cent of the normal) as affected by $La(NO_3)_3$. The normal rate represents a change in pH value from 7.78 to 7.60 in a number of seconds depending upon the amount of bacterial suspension used, usually 30 seconds. Average of three or four experiments; probable error of the mean less than 2 per cent of the mean.

Table I accompanies Fig. 2, showing the results at concentrations too small to be represented properly in the figure.

pure solution of the other component. The arrangement is made clear in the legend to the figure in each case by placing the words "left" and "right" after the component. Thus Curve A shows antagonism between NaCl (left) and $La(NO_3)_3$ (right) in concentrations of 0.8 M. A very small amount of $La(NO_3)_3$ sufficed to antagonize NaCl, the optimum proportion being 99.8 parts of NaCl to 0.2 parts of $La(NO_3)_3$.

Since in a previous investigation[1] it was found that one part of $CaCl_2$ was required to antagonize five parts of NaCl, it is evident that La is much more effective than Ca in antagonizing Na. This accords with the results of experiments by other investigators on

TABLE I.

*Rate of Respiration of Bacillus subtilis in Various Concentrations of Lanthanum Nitrate.**

Molecular concentration of $La(NO_3)_3$...	0.8	0.25	0.05	0.005	0.0005	0.00005	0.000025	0.0000062	0.0000025
Rate of CO_2 production.....	0	21	38	66	76	91	100	117	100

* Each number (representing the average of three or four experiments) expresses the rate (calculated as per cent of the normal) after an exposure of 1 hour. Probable error of the mean less than 3 per cent of the mean.

growth, length of life, electrical conductivity, etc. in which it is found that trivalent cations are more effective than bivalent in antagonizing monovalent cations.[3]

Curve B shows the effects of combinations of $CaCl_2$ (left) and $La(NO_3)_3$ (right) in concentrations of 0.8 M. There is a slight amount of antagonism which attains a maximum at 8 parts of calcium and 2 parts of lanthanum; from this point the curve approaches rapidly zero as the amount of lanthanum is increased.

[3] *Cf.* Loeb, J., The dynamics of living matter, New York, 1906. Osterhout, W. J. V., *Bot. Gaz.*, 1915, lix, 464.

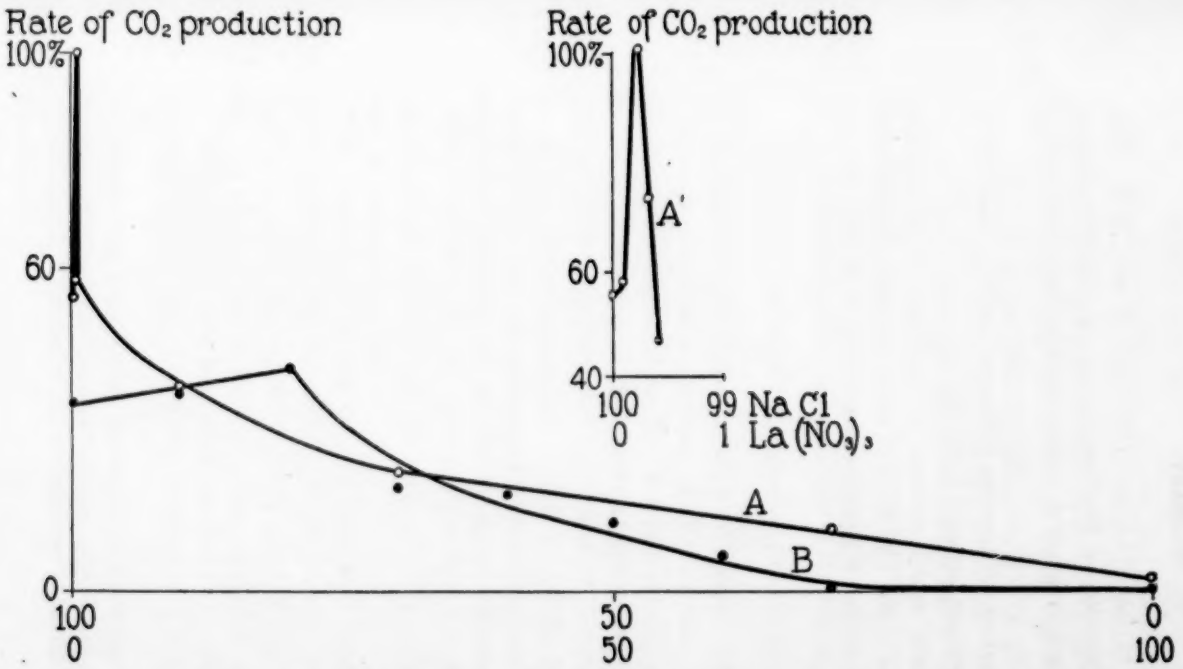

FIG. 3. Curve A shows antagonism between NaCl, 0.8 M (left) and $La(NO_3)_3$, 0.8 M (right). A portion of Curve A is represented on an enlarged scale (as A′) in the upper part of the figure. This was necessary because the ratios of the two salts in this region of the curve could not be shown accurately in Curve A.

Curve B shows a slight amount of antagonism between $CaCl_2$, 0.8 M (left) and $La(NO_3)_3$, 0.8 M (right).

The ordinates represent the rate of respiration (expressed as per cent of the normal); the abscissæ represent molecular proportions of the salts used. Thus in Curve A, the ordinate at the extreme left represents the rate in 0.8 M NaCl, while the ordinate at the extreme right represents the rate in 0.8 M $La(NO_3)_3$. The ordinate in the middle represents the rate in 50 parts of 0.8 M NaCl and 50 parts of 0.8 M $La(NO_3)_3$. The normal rate (which is taken as 100 per cent) represents a change in pH value from 7.78 to 7.60 in about 30 seconds, varying according to the number of bacteria used. Curve A, average of three experiments; probable error of the mean less than 2 per cent of the mean. Curve B, a single typical experiment.

SUMMARY.

1. Concentrations of $La(NO_3)_3$ up to 0.000025 M have little effect upon the rate of respiration of *Bacillus subtilis*; at 0.000006 M there is an increase in rate, while in higher concentrations there is a decrease in rate.

2. There is well marked antagonism between $La(NO_3)_3$ and NaCl, and very slight antagonism between $La(NO_3)_3$ and $CaCl_2$.

3. It requires a very small amount of $La(NO_3)_3$ to antagonize NaCl, the proportions of the two salts at their maximum antagonism being 99.8 parts of NaCl and 0.2 parts of $La(NO_3)_3$.

ON THE DIFFERENTIATION OF THE LEAF TISSUE FLUIDS OF LIGNEOUS AND HERBACEOUS PLANTS WITH RESPECT TO OSMOTIC CONCENTRATION AND ELECTRICAL CONDUCTIVITY.*

By J. ARTHUR HARRIS, ROSS AIKEN GORTNER, AND JOHN V. LAWRENCE.

(From the Department of Experimental Evolution and the Department of Botanical Research, the Carnegie Institution of Washington, Washington.)

(Received for publication, October 30, 1920.)

The existence of a differentiation of ligneous and herbaceous plants with respect to the magnitude of the osmotic concentration of the tissue fluids was first demonstrated in a strictly quantitative manner by work on the sap of the plants of the spring flora of the Arizona deserts[1] in the neighborhood of the Desert Botanical Laboratory, and on the terrestrial vegetation of the Jamaican montane rain forest.[2] These studies, in two geographically widely separated and climatically dissimilar regions, and an extensive series of unpublished observations demonstrate that the leaf tissue fluids of ligneous plants are characterized by an osmotic concentration materially higher than that of herbaceous forms.

The magnitude of the specific electrical conductivity, K, of the fluids must now be considered in comparison with osmotic concentration as measured by the freezing point lowering, Δ, for a series of plant species on which both of these constants were determined.

The determinations here considered were made on the north shore of Long Island during the spring and summer of 1914 and 1915. Leaf

* Studies carried out by the cooperation of the Department of Experimental Evolution and the Department of Botanical Research of the Carnegie Institution of Washington. The results will be published in full in the *Journal of Physical Chemistry.*

[1] Harris, J. A., Lawrence, J. V., and Gortner, R. A., *Phys. Researches*, 1916, ii, 1.

[2] Harris, J. A., and Lawrence, J. V., *Am. J. Bot.*, 1917, iv, 268.

tissue was collected in large test-tubes and squeezed as completely as possible after freezing to render the tissue permeable as has been shown to be necessary by Dixon and Atkins and by ourselves. The freezing point lowering, Δ, was determined in the usual manner. Correction was made for the ice separating on undercooling. The specific electrical conductivity, K, of the centrifuged sap was measured at 30°C. in a Freas conductivity cell, standardized by considering the conductivity of 0.1 N KCl to be 0.01412 reciprocal ohms, by means of the ordinary meter bridge wire.

The determinations for each species were then averaged and the deviation of each determination from the average for the species was calculated. All numbers which showed a deviation of more than ± 20 per cent for either Δ, K, or $\frac{K}{\Delta}$ were discarded, and a new average with

TABLE I.

Growth form.	Average. Δ	Average. $K \times 10^6$	Average. $\frac{K}{\Delta} \times 10^6$
Trees	1.292±0.043	11,213±494	9,092±462
Shrubs	1.177±0.024	10,770±339	9,529±372
Trees and shrubs	1.217±0.022	10,922±281	9,378±292
Herbs	0.846±0.011	14,308±192	17,674±282

deviations $< \pm 20$ per cent was determined. The statistical constants are, therefore, based on carefully selected averages whenever more than a single determination was available for a species. Determining the averages from the protocols of measurements we have the results for the three growth forms, and for a combination of the two groups of ligneous plants set forth in Table I.

The constants show that the tissue fluids of both trees and shrubs are characterized by a far greater freezing point lowering than are those of herbaceous plants. The mean freezing point lowering of the leaf tissue fluids is greater, although perhaps not significantly greater in comparison with its probable error, in arborescent than in shrubby species. The differences between trees and herbs, shrubs and herbs, and all ligneous plants and herbs are several times as large as the probable error of the difference and so unquestionably significant.

Expressing the differences in per cents of the values for ligneous forms, we note that the value for trees and shrubs is 30.46 per cent higher than that of herbaceous plants. These results are, therefore, in excellent agreement with those found in the Arizona deserts and in the Jamaican rain forest.

The constants for specific electrical conductivity show that the conductivity for shrubs is slightly lower than that for trees. The difference is, however, smaller than its probable error. The differences between the conductivities of the fluids of trees and herbs, shrubs and herbs, and both ligneous forms and herbs are several times as large as their probable errors and show that the conductivity is distinctly higher in herbaceous than in ligneous species.

The ratio of conductivity to freezing point depression is much smaller in both trees and shrubs than it is in herbs. The mean value of $\frac{K}{\Delta}$ is lower in trees than in shrubs, although the difference cannot be considered significant in comparison with its probable error. The ratios ($\times 10^6$) are 9,092:17,674 in the case of trees and herbs and 9,529:17,674 in the case of shrubs and herbs. Since the ratio does not differ significantly in trees and shrubs it is quite proper to combine them, and we obtain an average of $\frac{K}{\Delta}$ in all ligneous plants of 9,378 $\pm$ 292 as compared with 17,674 $\pm$ 282 in herbs. Thus the ratio $\frac{K}{\Delta}$ is about 90 per cent higher in herbaceous than in ligneous plants.

The foregoing results show clearly that the osmotic concentration is higher while the electrical conductivity is lower in the tissue fluids of ligneous than in those of herbaceous species. Because of the wide geographic range and the great diversity of conditions (xerophytic, mesophytic, and hygrophytic) under which the investigations on osmotic concentration were carried out there can be no reasonable doubt that the differentiation of ligneous and herbaceous plants with respect to the magnitude of their osmotic concentration is a general biological law. Until confirmed by investigations in other regions presenting different conditions for plant growth—investigations which are now in progress—the results for conductivity cannot be asserted to be of universal validity.

EXPERIMENTAL PRODUCTION OF GIGANTISM BY FEEDING THE ANTERIOR LOBE OF THE HYPOPHYSIS.

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Perhaps the most reliable information as to what may be the function of the hypophysis (or any other endocrine gland) may be expected to be obtained through experiments on the extirpation and transplantation of the gland. The majority of these experiments have shown in a rather conclusive way, that growth and development are inhibited in the partial (mammalians) or total (amphibians) absence of the anterior lobe; an increase of the rate of growth ensues if anterior lobes are grafted to the animals.[1] Particularly clear are the results obtained in amphibians. As shown by Smith[2] and by Allen,[3] the extirpation of the anterior lobe of the hypophysis results in an inhibition of growth and metamorphosis of the operated tadpoles. Recently Allen[4] has shown that grafting the anterior lobe of adult frogs on tadpoles causes an acceleration of growth and development in normal larvæ, and that it also restores the power of growth and development after they had been lost through extirpation of the anterior lobe.

These experiments seem to demonstrate that the anterior lobe of the hypophysis is the organ which makes growth possible during the normal growth period of life. They do not afford, however, any evidence as to whether the substance of the anterior lobe can cause growth to continue beyond the period of life in which, under normal conditions, the ability of growth is lost and whether in this way the anterior

[1] For a more complete discussion of the literature see Uhlenhuth, E., The rôle of the internal secretions in growth and development, in a book on Internal secretion and metabolism, edited by L. F. Barker and R. G. Hoskins (in press).

[2] Smith, P. E., *Science*, 1916, xlix, 280; *Anat. Rec.*, 1916–17, xi, 57.

[3] Allen, B. M., *Science*, 1916, xliv, 755.

[4] Allen, B. M., *Science*, 1920, lii, 275.

lobe substance can increase the size of the individual over the normal "maximum" size of the species. It is well known that growth of every individual stops as soon as the specific size of the species is reached. Many problems pertaining to this phenomenon would appear in a new light if it were possible to cause gigantism by a particular substance.

Clinical observations point to the conclusion that at least one form of gigantism is caused by an excessive production of anterior lobe substance; nevertheless, attempts to produce experimental gigantism have so far been unsuccessful. The only way to attack this problem seems to be the feeding of the anterior lobe substance by mouth. Such experiments have been attempted in large numbers but the results have for the most part been contradictory and difficult to interpret. The majority of investigators have merely desired to determine whether or not feeding of anterior lobe modifies in a specific way the rate of growth. It will be pointed out later that the greatest care is necessary in the interpretation of results obtained from feeding experiments. From the more recent feeding experiments, and especially those performed by Hoskins and Hoskins[5] and by Smith[6] on tadpoles, by Robertson[7] and his coworkers on white mice, and by Wulzen[8, 9] on chickens, most students of endocrinology have concluded that the anterior lobe substance retards growth in early periods of life, while later on it may cause an acceleration of growth.

But in these experiments none of the animals fed with anterior lobe developed into giants, except in two cases in which the slightly greater size of the experimental animals may have been due to the effect of the anterior lobe substance. Robertson and Ray[10] claim that they obtained unusually large mice, when the feeding of anterior lobe substance was started at an age of 4 weeks and discontinued at an age

[5] Hoskins, E. R., and Hoskins, M. M., *Endocrinology*, 1920, iv, 1.

[6] Smith, P. E., *Univ. California Pub., Physiol.*, 1918, v, 11.

[7] Robertson, T. B., *J. Biol. Chem.*, 1916, xxiv, 385, 397, 409. Robertson, T. B., and Delprat, M., 1917, xxxi, 567. Robertson, T. B., and Ray, L. A., 1919, xxxvii, 393, 427, 455.

[8] Wulzen, R., *Am. J. Physiol.*, 1914, xxxiv, 127.

[9] Wulzen, R., *J. Biol. Chem.*, 1916, xxv, 625.

[10] Robertson, T. B., and Ray, L. A., *J. Biol. Chem.*, 1919, xxxvii, 455.

of 12 weeks. In experiments in which the chicks were fed anterior lobe from an early stage, Wulzen[9] succeeded in raising one anterior lobe-fed cock which weighed 1,882 gm. as against the weight of only 1,597 gm. of the control animal; no normal cock raised by Wulzen grew to the size of the hypophysis-fed bird. Wulzen, however, raised only one cock of this kind; moreover, no records are given in the paper as to the normal maximum size of that race of chickens, and, therefore, it is not certain that this cock could be actually considered a true giant.

From the experiments to be reported in this paper, it will become evident that at least in one group of cold blooded animals, namely in salamanders, feeding of anterior lobe leads to the attainment of a size considerably in excess of not only the normal "average" size, but of even the greatest known size of the two species (*Ambystoma opacum* and *Ambystoma tigrinum*) employed in these experiments.

Critical Remarks Concerning the Methods of Feeding.

It is usually stated that in order to obtain reliable results in feeding experiments of this kind, only small amounts of the glandular product should be added to an otherwise normal diet. I, however, am of the opinion that this method, although considered at present as the standard method, cannot, at least under all conditions, give conclusive results. It is possible that a gland may contain a specific growth-promoting principle and yet may not give, with the above mentioned method, positive results if the amount of the hormone fed is too small. We do not know how much of a supposedly hormonic substance is contained in a definite amount of the fresh product, nor do we know how much of it may be required to produce gigantism or accelerated growth. While most hormones act in small quantities, it would be premature to conclude that all hormones should act in small amounts. The main point in experiments of this kind is to be able to prove that the control animals are fed in such a way as to show the maximal rate of normal growth known to exist in the species experimented upon.

In my experiments the controls were fed exclusively the normal diet, while the experimental animals were fed exclusively on the anterior lobes of the hypophysis of cattle. This method is based on the

following considerations. If earthworms are a complete diet for salamanders, the worm-fed animals should grow at the maximum rate characteristic of the species, provided that the calories and vitamines are in sufficient quantity to make the maximum rate of growth at that particular temperature possible. If by chance the anterior lobe should have a higher food value than the earthworm, this could not result in a better growth as long as the food value of the earthworm suffices for the maximum normal growth. If the anterior lobe of the hypophysis contains only ordinary food substances and no specific hormone for growth, the rate of growth of the animal should not exceed the specific maximum rate.

Hence only two conditions had to be provided for. First, all animals, controls and experimental, had to be kept at the same temperature, a condition which was carefully provided for. Second, all animals were given as much food as they desired to take. This condition was approached as nearly as possible.

The worm-fed animals took the worms readily and voluntarily. The gland-fed animals, however, had to be fed by pushing the pieces of gland into their mouths; yet it was possible to adjust the quantity of food according to the appetite of the animals. They yield readily if the food is gently pressed against their jaws, and swallow the food quickly when they are hungry; while they push the food back by means of the tongue or eject it when it is forced into the mouth, if they are not hungry.

The quantity of food taken up by the controls was generally greater than the quantity of hypophysis taken by the experimental animals.

Characteristics of Growth of Normal Salamanders.

Two species of salamanders were employed in these experiments, *Ambystoma opacum* and *Ambystoma tigrinum*. Since little is known about the normal growth of these animals, some data pertaining to it may be recorded here.

Large numbers of *Ambystoma opacum* were reared from eggs in my laboratory and the growth of the species has been recorded over long periods. The longest record I possess is that of four animals which at present are 3 years (161 weeks) of age and were raised from eggs of

two different females. Many animals of this species were observed for a period of over 2 years and behaved essentially the same way. Fig. 1 shows the growth curves of the four 3 year old animals and illustrates the most important characteristics of the growth of that species. Although each animal, during the larval period, was treated in a different way (D_1 normal, E_3 underfed, $W_{Ca}5$ kept in water + a small amount of Ca lactate, $W_{Na}4$ kept in water + a small amount of Na lactate) all four curves are about the same. The most rapid growth takes place during the larval period (first period of growth); at the end of this period a decrease in size is noticeable which may last for several weeks and corresponds to metamorphosis. The second period of growth lasts from after metamorphosis till the first breeding season, at which the animals are about 1 year old. The beginning of the breeding season in the male may be recognized by the swelling and reddening of the cloacal region and in both males and females by the cessation of food intake. During the second period of growth the animals continue to grow at a fairly high rate; during the first as well as the following breeding seasons little growth or even a decrease in size may be noticed. The third period of growth begins after the first breeding season and lasts till the third breeding season, and is characterized by slow growth. No records are available to determine the behavior after the third breeding season, but to conclude from the normal maximum size of the species growth seems to be nearly completed at the end of the third period. The control animals of the hypophysis-fed series exhibit a similar type of growth (Fig. 2), although they were from a different season (1918). It is safe to assume that this is the type of growth characteristic for the species *Ambystoma opacum*. The most important feature of it, in connection with the following experiments, is the considerable slowing down of growth after the first breeding season.

As to the normal size of the species, the following records are available. The largest animal on record in my laboratory was 115 mm. long at an age of 79 weeks (it was one of the controls of the hypophysis-fed animals). The largest animal among fifteen specimens of from 2 to 3 years of age, still alive at present, measures 113.5 mm., the average size of these fifteen animals being 103.5 mm. (the small size of some of them may be due to the abnormal conditions under

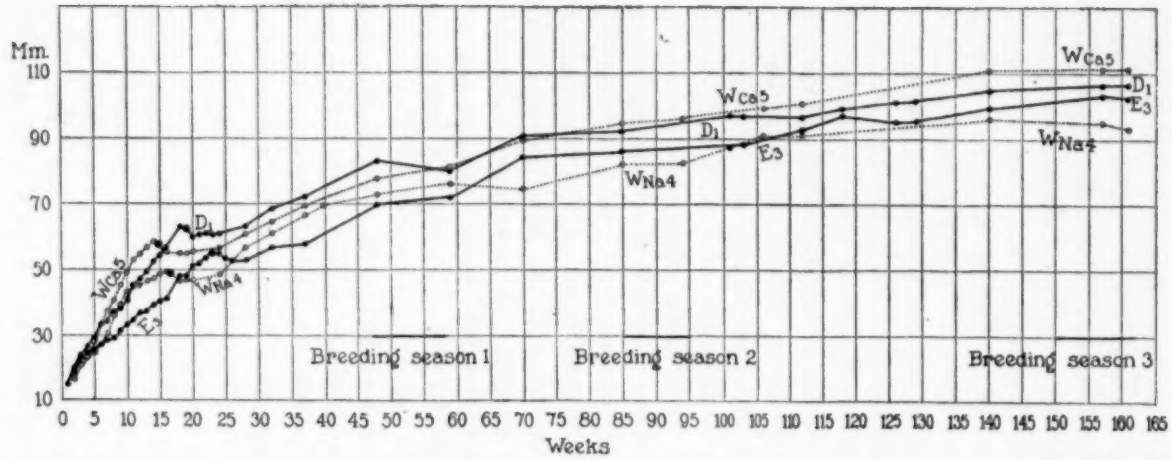

FIG. 1. Growth of four specimens of *Ambystoma opacum* during a period of 3 years. All four animals were fed normal diet, but E_3 was underfed during the larval period and W_{Ca} 5 and W_{Na} 4 were kept in water + small amounts of Ca lactate and Na lactate respectively during the larval period. The figure is drawn to a smaller scale than Figs. 2, 3, 4, and 5.

which they were kept during the larval period). Two breeding females, collected recently outdoors, measured 112 mm. and 106 mm. respectively.[11] Cope,[12] in his book on North American batrachians, mentions the specimen, from which he described the species, as measuring 3.8 inches (about 100 mm.). The largest animal found in the collection of the American Museum of Natural History[13] measured 117.7 mm.; the average of the eight largest animals was 100.3 mm.

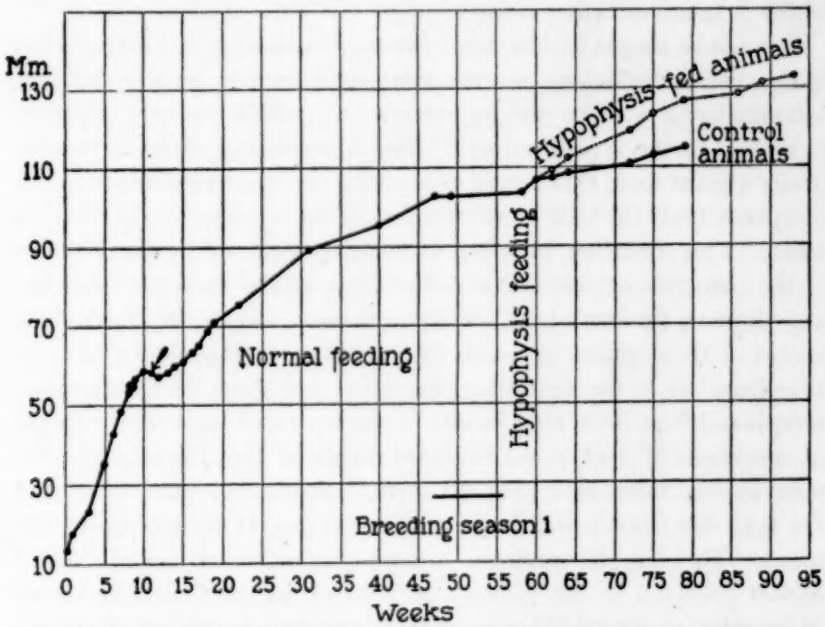

FIG. 2. Experiment 1 (*Ambystoma opacum*, 1918, Series XIV). The unbroken line indicates the growth of the controls, the dotted line that of the anterior lobe-fed animals.

To conclude from these data the maximum size of the species is nearly 118 mm., while specimens measuring more than 115 mm. are of very rare occurrence.

[11] I am obliged to Mr. George P. Engelhardt, of the Brooklyn Museum, for this record.

[12] Cope, E. D., The batrachia of North America, Washington, 1889.

[13] I wish to express my appreciation to Miss M. Dickerson for giving me the opportunity of examining the collection of salamanders at the American Museum of Natural History.

I have no other records concerning the growth of the species *Ambystoma tigrinum* except those afforded by the control animals of these experiments. These give information on the growth during a period of only 1½ years (84 weeks), the present age of the animals of Series A, XLVI, and LV. Apparently this species, as illustrated in Figs. 3, 4, and 5, behaves very much in the same way as the species *Ambystoma opacum*, growth being most rapid during the larval period, and continuing at a fairly high rate till about the end of the 1st year, after which it becomes rather slow.

It is not so simple to determine the maximum size of this species as in the case of *Ambystoma opacum*, since there are two races of the tiger salamander, a western and an eastern one, which are very different in respect to the type of growth. The western race seems to be naturally a giant race; this is true at least for the specimens which metamorphose from the well known neotenous larvæ found in the western lakes. This condition, however, is doubtlessly caused by disturbances of the endocrine system and is rather pathological than normal. My experiments, performed with eastern animals, cannot be checked by means of these giants although they are apt to throw some light on the gigantism of the neotenous specimens and those that have metamorphosed from neotenous larvæ. Cope in describing a large number of specimens of *Ambystoma tigrinum* mentions that the largest specimen among them measured 10 inches (about 244 mm.) and that De Kay described a still larger one measuring 11 inches (about 280 mm.). The largest specimen among 55 neotenous larvæ which I myself collected in the vicinity of Tolland, in the Colorado Rocky Mountains, measured 257 mm., while all the metamorphosed specimens collected in the same locality were much smaller. As pointed out above, I have used in my experiments only eggs that were from females of the eastern race. Unfortunately, I have not reared enough animals of this species to form a conclusive idea as to the normal maximum size of the species. But the largest individual on record in my laboratory (one of the controls of these experiments) measures 200 mm. at an age of 84 months; it is, however, still growing, although very slowly. In the collection of the American Museum of Natural History I found the largest specimen among the nine largest animals, eastern as well as western, to measure 208.7 mm.

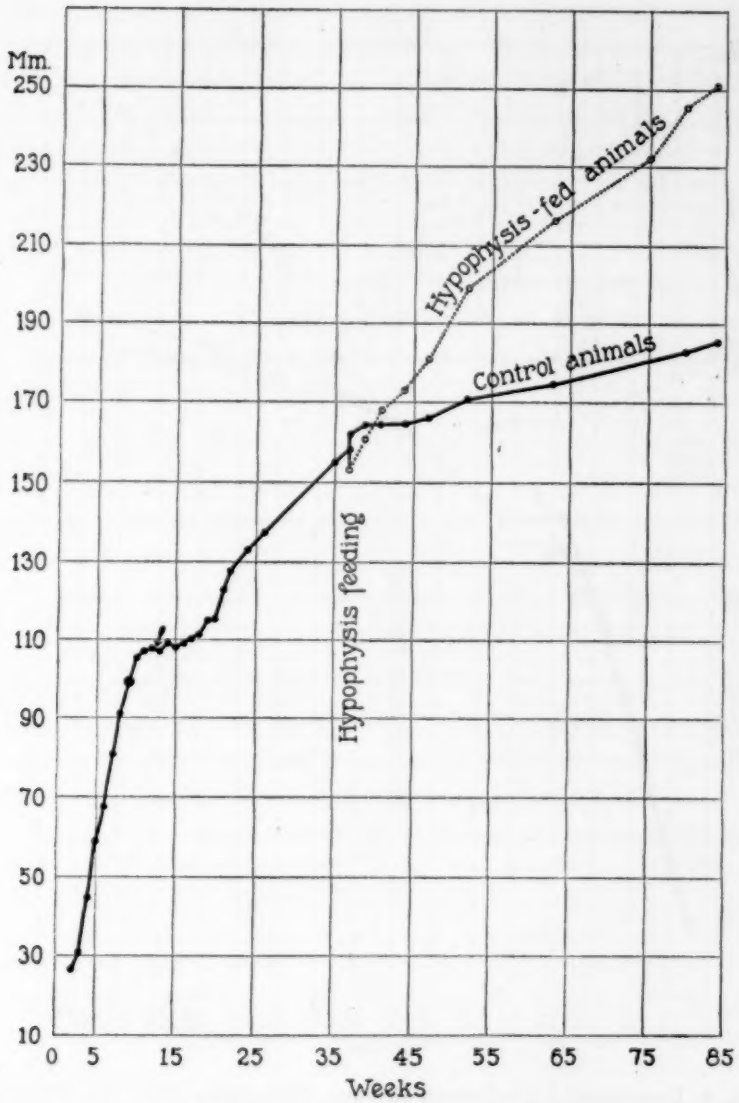

FIG. 3. Experiment 2 (*Ambystoma tigrinum*, 1919, Series XLVI). The unbroken line illustrates the growth of the controls, the dotted line that of the anterior lobe-fed animals.

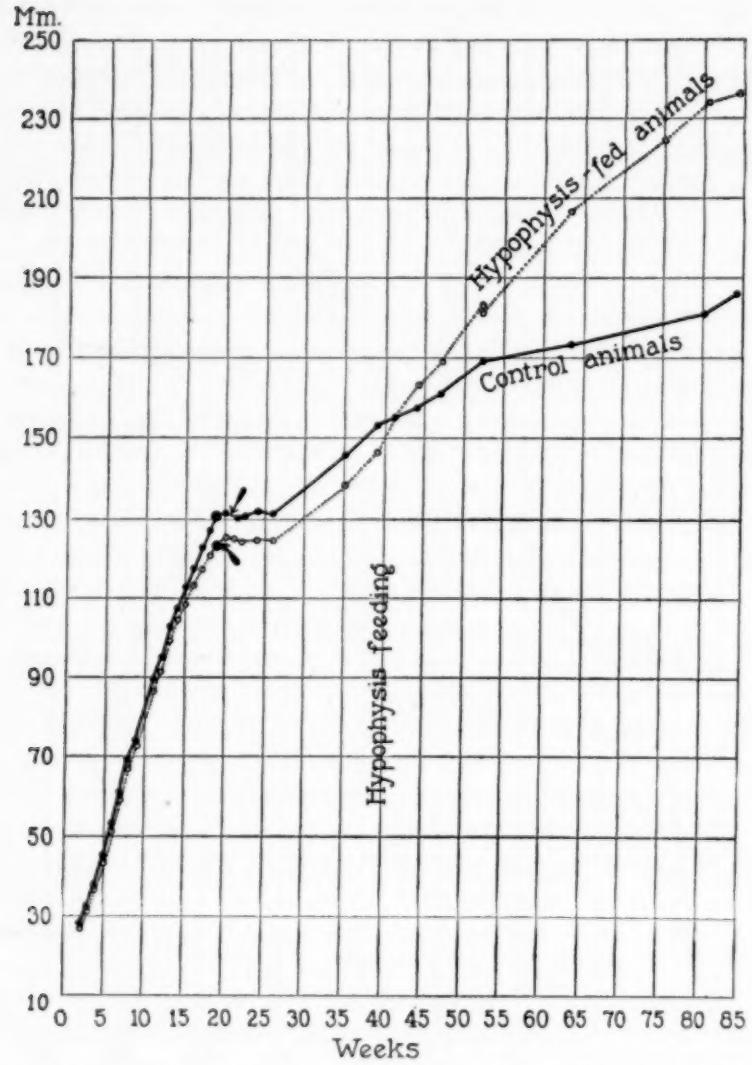

FIG. 4. Experiment 3 (*Ambystoma tigrinum*, 1919, Series LV). The unbroken line represents the growth of the controls, the dotted line that of the anterior lobe-fed animals.

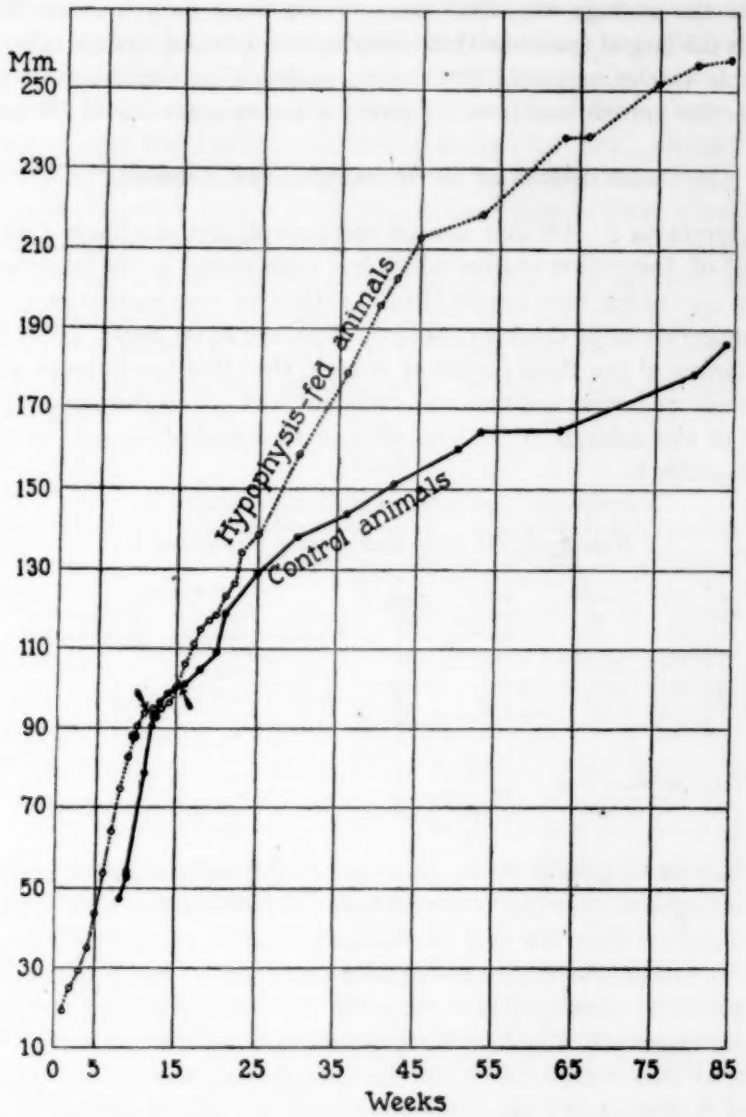

FIG. 5. Experiment 4 (*Ambystoma tigrinum*, 1919, Series A and XXXVII). The unbroken line represents the growth of the controls, the dotted line that of the anterior lobe-fed animals.

while the average was 200.8 mm. From these data it seems that while the largest specimen that could be found among eastern animals of this species measured 208.7 mm., pathological conditions of the endocrine system may produce giants of a maximum size of 280 mm.

The Growth of the Hypophysis-Fed Animals.

Experiment 1.—Of four normal metamorphosed specimens (Series XIV) of *Ambystoma opacum* which had been reared in the laboratory from eggs of the same female (Brood 1918) two were started on a diet of anterior lobe of cattle hypophysis at an age of 60 weeks, *i.e.* at the beginning of the third period of growth; the other two animals were controls and were kept on an abundant diet of earthworms. The size of the animals at the beginning of the experiment may be seen from Table I.

TABLE I.

Hypophysis-Fed Ambystoma opacum, Experiment 1.

	Animal No.	Size of animals.		
		60 weeks.	72 weeks.	End of experiment.
		mm.	mm.	mm.
Control	4	102.1	108.1	108.1 at 72 weeks.
	1	111.0	113.2	115.0 " 79 "
Anterior lobe-fed animals	2	110.4	120.6	130.5 " 105 "
	3	103.1	118.8	138.0 " 101 "

The rate of growth of the anterior lobe-fed animals, after 2 weeks of feeding, rose over that of the controls and continued at such a height as is usually observed only in the second period of growth (Fig. 2). The two control animals, although they were fed on an abundant diet, continued to grow at a slow rate (Fig. 2) characteristic of the third period of growth (Fig. 1). In consequence of their rapid growth the anterior lobe-fed animals, 12 weeks after the beginning of the experiment, had reached a size exceeding that of the largest animals of this species on record (Table I, 72 weeks). At the end of the experiments (caused by the death of the animals) the largest anterior lobe-fed animal measured 138 mm., the smaller one 130 mm., while the controls had reached a size of only 115 and 108 mm. respectively. Unfortu-

nately both of the controls were lost before the anterior lobe-fed animals had reached such an extraordinary size, but from our experiences with many other animals as well as from the maximum size of this species it is safe to conclude that the growth of the controls in this experiment was nearly completed. On the other hand, the size of the anterior lobe-fed animals exceeded the normal maximum size of the species so much as to leave no doubt that both of them were giants. The size of the larger experimental giant exceeded the size of the largest normal animal of this species on record by 20 mm., and the size of the smaller experimental giant exceeded the largest normal animal on record by 12 mm. It is certain that both animals would have become still larger had they survived, since they were still growing before they died.

TABLE II.

Hypophysis-Fed Ambystoma tigrinum, Experiment 2.

	Animal No.	37 weeks.	63 weeks.		84 weeks.	86 weeks.
		Size.	Size.	Weight.	Size.	Weight.
		mm.	mm.	mg.	mm.	mg.
Controls	2	152.4	163.0	22,400	171.5	34,800
	3	163.5	183.0	31,200	190.0	40,000
	4	167.9	179.0	27,900	197.0	35,200
Anterior lobe-fed animals	5	163.5	233.0	57,200	268.0	72,300
	6	142.5	199.0	50,500	233.0	78,000

Similar results were obtained when metamorphosed specimens of the species *Ambystoma tigrinum* were fed on anterior lobe.

Experiment 2.—Five larvæ (Series XLVI) were reared from eggs of the same female and fed on earthworms. At the end of the second growth period, about 24 weeks after metamorphosis, when the animals were 37 weeks old, three of them, among them the largest one of the series, were kept on earthworms, while two, among them the smallest one, were started on an exclusive diet of anterior lobe (for initial sizes see Table II).

Although the control animals were fed on an abundant supply of earthworms, their rate of growth remained low as compared with that during the second period of growth. The animals fed on anterior

lobe, however, commenced to grow so rapidly that their rate of growth soon exceeded that which prevailed even during the earlier period of life (see Fig. 3). At an age of 63 weeks, 26 weeks after the beginning of the experiment, the smallest animal fed on anterior lobe was 20 mm. larger than the largest control animal, although at the beginning of the experiment it was 10 mm. smaller than the smallest control specimen; its weight was more than twice the weight of that of the smallest control animal. At an age of 84 weeks the largest animal of this series fed on anterior lobe measured 268 mm. as compared with 197 mm., the latter being the size of the largest control animal. Hence the size of the largest anterior lobe-fed animal exceeds at present the size of the largest normal animal, on record in the laboratory, by 68 mm. and the size of the largest specimen of the eastern race of *Ambystoma tigrinum* by 60 mm. It is 12 mm. smaller than the largest specimen of the western race on record, which, however, cannot be considered normal, as pointed out above. Both the controls and the anterior lobe-fed animals are still growing and at the present time the rate of growth of the anterior lobe-fed specimens is still higher than that of the control animals (see Fig. 3). It is possible, therefore, that in a short time, the largest anterior lobe-fed animal will exceed in size even the largest western specimen known to the writer.

Experiment 3.—Six larvæ (Series LV), of the same brood from which the animals of Experiment 2 were taken, were raised on earthworms. At the end of the second period of growth, about 19 weeks after metamorphosis and at an age of 39 weeks, three of them (Nos. 1, 2, and 4), among them the largest one, were used as worm-fed controls; the other three animals, among them the smallest one, were kept on a pure anterior lobe diet (Table III). The largest of these latter animals died soon after commencement of the experiment.

The results were similar to those obtained in Experiment 2 and may be seen from Table III and Fig. 4. At an age of 84 weeks the larger one of the two hypophysis-fed animals measured 51 mm. more than the largest control animal, and 44 mm. more than the largest animal of the eastern race of the species known to the writer. The hypophysis-fed specimens as well as the control animals are still growing, but at present the growth of the anterior lobe-fed specimens of this series is less vigorous than that of the controls.

Experiment 4.—In this experiment (Series XXXVII$_2$ and Series A) the controls were underfed and therefore cannot be used for comparison. Both the experimental as well as the control series were obtained from the eggs of the same female; but the control animals were underfed for several weeks during the larval period and the experimental animal, which was fed abundantly on anterior lobe from the beginning of its life, was kept in distilled water during the larval period, which tends to retard growth. The results are seen in Fig. 5. The anterior lobe-fed animal measured 258 mm. at an age of 84 weeks; *i.e.*, 58 mm. more than the largest normal animal kept in the laboratory and 50 mm. more than the largest specimen of the eastern race. At present, however, it is growing very slowly and less vigorously than the animals of Series A.[14]

TABLE III.

Hypophysis-Fed Ambystoma tigrinum, Experiment 3.

	Animal No.	39 weeks.	63 weeks.		84 weeks.	86 weeks.
		Size.	Size.	Weight.	Size.	Weight.
		mm.	mm.	mg.	mm.	mg.
Controls.....	1	163.8	182.2	27,400	200.2	38,500
	2	156.1	176.4	30,000	182.0	40,200
	4	137.2	161.5	22,500	176.5	31,100
Anterior lobe-fed animals.....	3	136.4	208.5	54,200	251.6	95,700
	5	146.7	204.0	60,000	221.5	84,300
	6	156.1	Dead.			

[14] Since this paper went to press, Dr. Leonhard Stejneger, head curator of the United States National Museum, was kind enough to go over the entire collection of the museum and let me have the measurements of the largest specimens of the species *Ambystoma opacum* and *Ambystoma tigrinum*. Since the measurements are based on a far larger collection of material than my own data reported in the preceding pages, they are of greater importance and will be added below. They confirm the opinion expressed in this article that feeding the anterior lobes of hypophysis produces gigantism in salamanders.

Ambystoma opacum.

Normal.	Hypophysis-fed.
mm.	mm.
119	138
116	130

DISCUSSION.

The feeding of anterior lobe to metamorphosed salamanders has two different effects on the growth of the animals: first, it increases the rate of growth over that of normal animals; and, second, it maintains growth after the normal "maximum" size of the species has been attained. Have we any reason to consider these effects as the result of a "specific" growth-promoting hormone?

As to the first point, we cannot be quite certain that the rate of growth of our control salamanders was the highest rate of growth which can be obtained with a normal diet. Unfortunately the data available on the growth of metamorphosed salamanders are not numerous enough to decide if earthworms form a sufficiently complete diet for the two species in question. Although it is quite certain that these animals do not require a plant diet, not enough food materials have been tested by the writer to state on which food they nat-

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The two largest hypophysis-fed specimens of *Ambystoma opacum* exceed in size the largest known animal of this species by 19 and 11 mm. respectively.

Ambystoma tigrinum.

Eastern race.

Normal.	Hypophysis-fed. (Age 88 weeks.)
mm.	mm.
235	273.5 (Experiment 2, No. 5)
223	235.5 (" 2, " 6)
	257.1 (" 3, " 3)
	226.2 (" 3, " 5)
	263.0 (" 4, " 2)

Among the five hypophysis-fed specimens of *Ambystoma tigrinum* three are considerably larger than the largest known specimens of the eastern race, the largest hypophysis-fed specimen exceeding in size the largest eastern specimen by 28.5 mm.

There is, as stated above, a western giant race of *Ambystoma tigrinum*. Dr. Stejneger gives 258, 265, 285, 285, and 292 mm. as measurements of the five largest specimens of this race. The largest of our hypophysis-fed animals, which has grown 5.5 mm. in the last 4 weeks and measures at the time of writing 273.5 mm., has already outgrown two of the western giants and will soon outgrow the three others if it continues to grow at the present rate.

urally grow best. Feeding of calf thymus or posterior lobe of hypophysis of cattle does not induce any growth equal to that of worm-fed animals. In the feeding of salamander larvæ I have used many different substances (frog muscle, beef muscle, lymph gland, parathyroids, thymus, spleen, cheese, milk) with and without the addition of normal food; it is certain that in larvæ no other diet can produce a rate of growth higher than that produced by earthworms. But, of course, the metabolic processes involved in growth may be quite different for the larvæ and the metamorphosed animals.

On the other hand, if we look at the curves of the various series, it is noticeable that the curves for the worm-fed animals are very much like the normal growth curves of most other animals whose growth has been studied carefully. In particular they show the gradual flattening out of the growth curves of warm blooded animals. The curves of the anterior lobe-fed animals, especially those of *Ambystoma opacum* (Fig. 2), with their sudden rise above the flat level of the normal curve, differ from this general normal type.

Although at present we must postpone more definite conclusions, it seems at least probable that the rate of growth of the animals fed exclusively on anterior lobe is the result of a specific growth-promoting hormone contained in the anterior lobe of the hypophysis.

It is beyond doubt that the size of the hypophysis-fed animals exceeds the "maximum" size of the species. The animals fed on anterior lobe are true giants. The hormone of the anterior lobe is not only able to accelerate growth, but also—and this is of far greater importance—possesses the property of maintaining growth when the normal size of the species has been reached. The production of experimental gigantism by means of feeding anterior lobe proves that this organ contains actually a specific substance which can overcome the obstacles which are responsible for the discontinuation of growth when the normal size is reached.

These experiments do not give any clue as to whether the cells of the body are directly affected by the hormone or whether this hormone acts by the intermediation of another organ. In view of the results obtained with single cells, it is probable that the cells are not directly affected by the anterior lobe hormone. It was found by Shumway, by

Chambers, and by Nowikoff[15] that the division rate of Protozoa is not increased if anterior lobe extract is added to the culture medium. The same is true for the cells of warm blooded animals. In as yet unpublished experiments Carrel found that in tissue cultures the growth of the cells of warm blooded animals cannot be accelerated if anterior lobe extract is added to the culture medium.[16] Our experiments show, however, that the cells of the organism are still capable of dividing at a time at which the organism as a whole has stopped to grow. Apparently the cessation of growth of the organism as a whole is not caused by a fundamental property of the cell to become incapable of growth, after growth has been going on for some time. This corroborates the well known tissue culture experiments of Carrel which would suggest that the cell protoplasm, if kept under proper conditions, can go on dividing for indefinite periods. Since the hypophysis-fed salamanders are still growing, it is impossible, at present, to say how long the anterior lobe hormone can maintain, within the organism as a whole, a condition permitting active growth of the cell. But it is possible to say that the size of the individual is not an obstacle to the further growth of an organism. It has frequently been claimed that the size of every species is determined by the mechanics and statics of the substances constituting the body of the organism. Although these principles may be among the reasons why the organism stops growing after it has attained a definite size, they cannot be the only reason; the occurrence of giants shows that at least a considerable increase in the specific size of the species would result if the size of the organism would be determined by the mechanic and static principles alone.

As pointed out above, the evidence that anterior lobe feeding can produce gigantism in warm blooded animals is not yet sufficient. It would be of great importance if it should be demonstrated that this difference is due to the difference in the body temperature of amphibians and warm blooded animals. Lenz[17] has recently called atten-

[15] Shumway, W., *J. Exp. Zool.*, 1917, xxii, 529. Chambers, M. H., *Biol. Bull.*, 1919, xxxvi, 82. Nowikoff, M., *Arch. Protistenk.*, 1908, xi, 309.

[16] I am indebted to Dr. Alexis Carrel for permission to quote these, as yet unpublished, experiments, which were carried out in Dr. Carrel's laboratory.

[17] Lenz, F., *Münch. Med. Woch.*, 1919, lxvi, 992.

tion to the fact that in acromegaly the tendency to abnormal growth is confined to the prominent parts of the body, in which the temperature is lower than in the main body.

SUMMARY.

1. Metamorphosed salamanders of the species *Ambystoma opacum* and *Ambystoma tigrinum* were fed on a pure diet of the anterior lobe of the hypophysis of cattle; the controls were fed on an abundant diet of earthworms.

2. The rate of growth of the animals fed on the anterior lobe of the hypophysis was greatly increased over the rate of growth of normal animals.

3. Growth of the animals fed on anterior lobe did not cease after they had reached the normal "maximum" size of the species, and experimental giants were produced.

4. The largest animal of the species *Ambystoma opacum* fed on anterior lobe of the hypophysis was 19 mm. larger than the largest normal animal of this species known to the writer; the largest animal of the species *Ambystoma tigrinum* fed on anterior lobe is at present about 28 mm. larger than the largest normal animal of the eastern race of this species known to the writer.

TIME AND INTENSITY IN PHOTOSENSORY STIMULATION.

By SELIG HECHT.

(From the Physiological Laboratory, College of Medicine, Creighton University, Omaha.)

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I.

The photochemical effect of light depends upon its intensity and the time of its action. In previous studies I have investigated the separate effects of these two variables in their action on the photic responses of *Mya arenaria* (Hecht, 1918–19, *a*; 1919–20, *c*). I have also considered the interaction of these two factors under certain specially circumscribed conditions of particular interest (Hecht, 1919–20, *b*, *c*). In its more general bearings, however, the relation between the two variables has not been examined. This I propose to do now.

If the intensity of the stimulating light is kept constant and its time of action varied, it has been found (Hecht, 1918–19, *a*) that the photochemical effect (E) is a linear function of the time (t). This may be written

$$E = k_1 t \quad (1)$$

where k_1 is a constant of proportionality. On the other hand, if the time of action is kept constant and the intensity varied, the photochemical activity of the light is found (Hecht, 1919–20, *c*) to be directly proportional to the logarithm of the intensity (I). This may be expressed as

$$E = k_2 \log I \quad (2)$$

k_2 being a constant.

It follows from equations (1) and (2) that if both the intensity and the time are permitted to vary, the photochemical effect should be proportional to the product of the time of action into the logarithm of the intensity. In other words

$$E = kt \log I \quad (3)$$

the terms having their previous significance.

If equation (3) is true experimentally, it should furnish a corroboration of the previous findings as expressed in equations (1) and (2). Moreover, it would then give substantial support from a new angle to the hypothesis which I have proposed to account for the photosensory behavior of *Mya* (Hecht, 1918–19, *a*). I have therefore undertaken a number of simple measurements which will give an unequivocal answer to the requirements of equation (3).

II.

An outstanding characteristic of the response of *Mya* to light is its reaction time. It has already been repeatedly shown in these studies on the photosensory process that this reaction time is composed of two parts, the sensitization period and the latent period (Hecht, 1918–19, *a*; 1919–20, *b*). The sensitization period represents the actual time of action (t) of the light in its relation to the reversible photochemical reaction

$$S \rightleftharpoons P + A$$

in the sense organ. The latent period is the portion of the reaction time during which the light is not required. It is occupied by the time taken for the secondary reaction

$$L \rightarrow T$$

to produce the amount of T necessary to initiate the nervous impulse which begins the response of the animals.

The velocity of the latent period reaction is directly proportional to the photochemical effect produced by the initial photochemical reaction. A constant photochemical effect gives a constant latent period. During ordinary stimulation of *Mya* by light, the latent period is constant and of minimal duration because the photochemical effect produced during the sensitization period is constant and maximal. The sensitization period, and consequently the reaction time, simply prolongs itself until the required accumulation of P and A is produced by the light.

The duration of the latent period cannot be decreased beyond this point because the velocity of the reaction

$$\mathrm{L} \rightarrow \mathrm{T}$$

is maximal when the amount of P and A has been produced during the exposure occupied by the sensitization period. It is, however, possible to *increase* the latent period by exposing the animal for periods less than the sensitization period. Submaximal quantities of P and A are then produced and the latent period is prolonged, because the velocity of the latent period reaction is diminished. If merely a minimum exposure is given (presentation time of Laurens and Hooker, 1920) the amount of P and A produced is minimal, and the latent period is consequently very much prolonged. In any event, however, a given photochemical effect results in a constant duration of the latent period.

These findings have been secured by such a variety of methods, and have been demonstrated so frequently to investigators at Woods Hole, that it is somewhat surprising to have them questioned (Laurens and Hooker, 1920). The experiments to be described will furnish still another means of corroboration, sufficient, I hope, to disarm any criticism.

The photochemical effect of the light is maximal if it takes place during the sensitization period. An animal which is exposed to light indicates automatically when this maximum has been reached by the retraction of its siphon at the end of the reaction time. Because the latent period is constant, variations in the reaction time to lights of different intensities are the result of variations in the sensitization period. This then furnishes a simple method of testing equation (3). All that is required is to measure the reaction time of animals exposed to lights of different intensities.

III.

The intensity of illumination may be varied by placing the animal at different distances from a source of light. As sources of light I have used on different occasions a 40 watt, a 100 watt, and a 250 watt lamp. These are concentrated-filament Mazda lamps, and for our purposes may be considered as point sources. The intensity of illumination therefore varies inversely as the square of the distance from the source. The reaction time is measured with a stop-watch.

Only dark-adapted animals are used; and between tests they are kept in complete darkness. At least 15 minutes are allowed between tests.

At different times during the summer I performed seven series of experiments: one with three animals; two with four animals; two with six animals; and two with seven animals. As an example, Fig. 1 gives the data of the series of experiments performed on July 28.

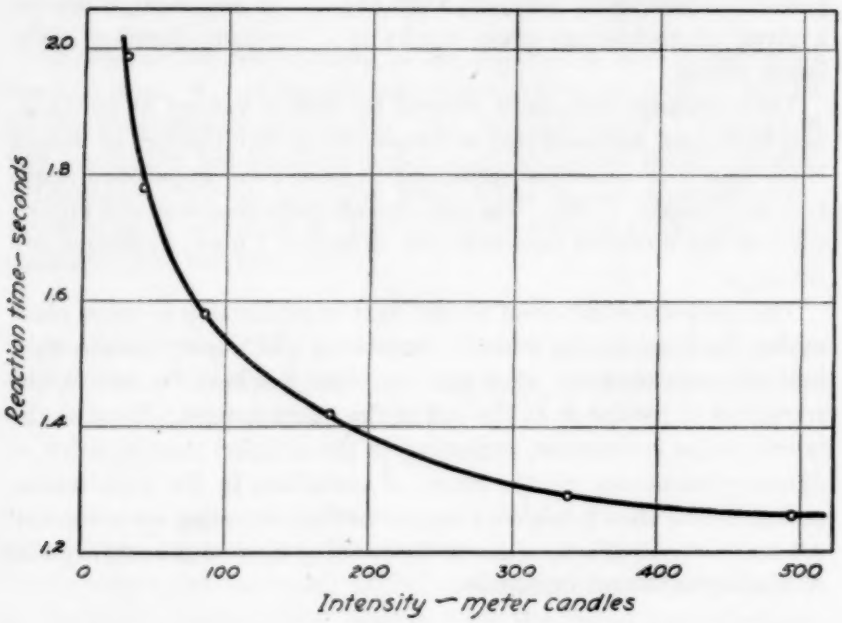

FIG. 1. Relation between the reaction time and the intensity of the stimulating light. Experiment 9.28; 40 watt lamp; six animals; three readings for each animal for every intensity.

The reaction time of each animal was measured at three different times for every intensity. Each point in Fig. 1 is thus an average of eighteen measurements, three with each of six animals. It is apparent that the experimental results are regular and may therefore be treated mathematically.

IV.

Equation (3) states that

$$E = kt \log I \quad (3)$$

For purposes of experimental verification, and for convenience in presenting the data, it will be desirable to consider equation (3) as follows. The reaction time (r) is equal to the sum of the time occupied by the sensitization period (t) and the latent period (p). Therefore

$$r = t + p$$

and

$$t = r - p \quad (4)$$

Substituting this value of t in equation (3) we get

$$E = k(r-p) \log I$$

and from it that

$$r = K \frac{E}{\log I} + p \quad (5)$$

where $K = \frac{1}{k}$. It is at once apparent that equation (5) is in the form of the equation of a straight line

$$y = ax + b$$

where $y = r$, or the reaction time; $a = K$; $x = \frac{E}{\log I}$, which is equivalent to the reciprocal of the logarithm of the intensity, since E, the photochemical effect, is constant; and $b = p$, the latent period.

It follows from this consideration that if equation (3) is true, a curve, representing as ordinates the reaction time (r) and as abscissæ the reciprocal of the logarithm of the corresponding intensity $\left(\frac{1}{\log I}\right)$, should be a straight line which crosses the y axis at p units above (0,0).

I have drawn the data of Fig. 1 in this way, and the result is given in Fig. 2. The fact that the curve in Fig. 2 is a straight line is direct proof of the experimental validity of equation (3) and of its derivative, equation (5). The data of the other six series of experiments show precisely the same thing. For further emphasis Fig. 3 is presented, which gives the data of two other experiments. The three

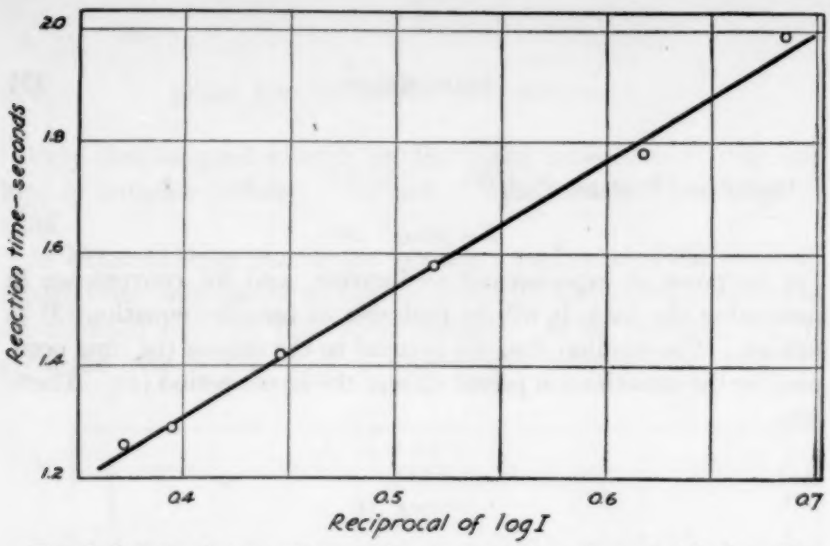

FIG. 2. Relation between the reaction time and the reciprocal of the logarithm of the intensity. Same experiment as Fig. 1.

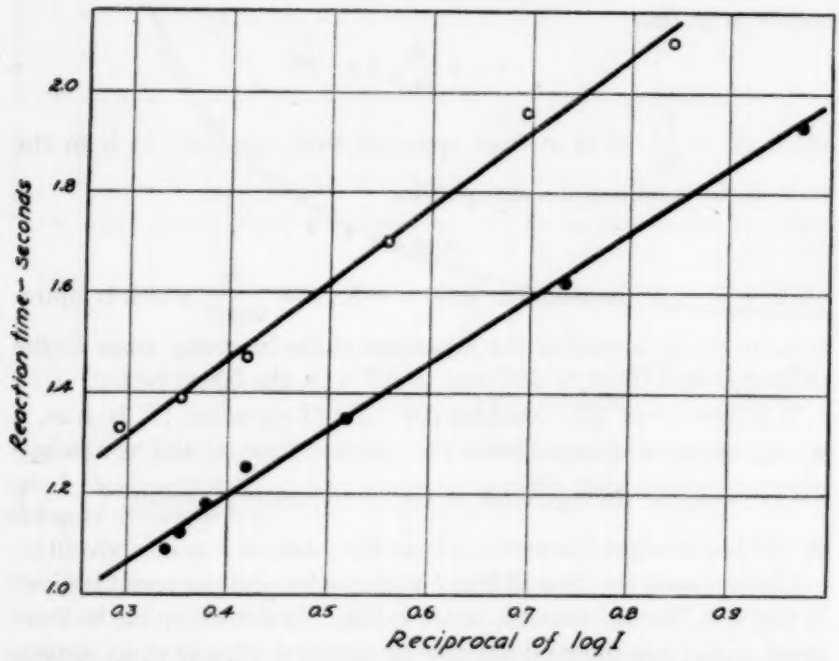

FIG. 3. Relation between the reaction time and the reciprocal of the logarithm of the intensity. Open circles are the data of Experiment 9.9; 250 watt lamp; seven animals; one measurement for each animal for every intensity. Dark circles are the data of Experiment 10.10; 100 watt lamp; seven animals; three readings for each animal for every intensity.

groups of experiments shown in the two figures were made at intervals of about 3 weeks, with different sources of light, and, of course, with different animals.

There can therefore be no doubt that equations (3) and (5) are true experimentally. The photochemical effect of light in the photosensory stimulation of Mya is then proportional to the product of the logarithm of the intensity of illumination into the time during which the light acts. This result supports previous investigations with *Mya* (*cf.* especially Hecht, 1919–20, *c*), and strengthens the hypothesis proposed to account for its photosensory responses.

SUMMARY.

In its photosensory effect, the action of light depends on two variables,—intensity and time. If the intensity alone is varied, the photochemical effect is proportional to the logarithm of the intensity. If the time alone is varied, the effect is proportional to the time. Experiments here reported show that when both the intensity and the time are varied, the photochemical effect is equal to the product of their separate activities: $E = kt \log I$. These results furnish the means of expressing directly the relation between the intensity of illumination and the reaction time of *Mya*.

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THE RELATION BETWEEN THE WAVE-LENGTH OF LIGHT AND ITS EFFECT ON THE PHOTOSENSORY PROCESS.*

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I.

1. In a series of publications I have described several phases of the mechanism which underlies the sensitivity of certain animals (*Mya* and *Ciona*) to light (Hecht, *a*, *b*, *c*, *d*). The basis of this mechanism is essentially a process involving two intimately connected chemical reactions. The first of these is a reversible photochemical reaction

$$S \underset{\text{"dark"}}{\overset{\text{light}}{\rightleftharpoons}} P + A$$

in which S is the sensitive substance, P the principal product of decomposition, and A its accessory. The second reaction is an ordinary chemical reaction

$$L \rightarrow T$$

which is catalyzed by the products of reaction of the photochemical reaction. The thermolabile material T is the end-product of the sensory process, and a definite quantity of it is required for the initiation of an impulse which results in the response of the animal to illumination.

The properties of these different substances and reactions are derived from quantitative studies of the responses of animals under conditions of experimentation calculated to bring them out. As the characteristics of the reactions and their components become known

* These experiments were carried out with the aid of a grant from the Elizabeth Thompson Science Fund, to the trustees of which I wish here to express my thanks.

in greater detail, their identification becomes increasingly possible. Heretofore attention has been centered mainly on the kinetic and dynamic relations of the sensitive system. It is the purpose of the present observations to furnish the basis for the description of a physical property of the photosensitive substance *S*.

2. It is axiomatic to say that in a photochemical transformation only the light which is absorbed is active in producing chemical change. Although the converse,—that all the light which is absorbed gives rise to chemical action,—is not proved, it is well known that the absorption spectrum of a chemical system is intimately connected with its photochemical behavior (Sheppard, 1911, p. 140). Thus, if in a given photochemical system we know the relative effectiveness of a series of lights of different wave-length, it is possible to draw certain conclusions with regard to the absorption spectrum of the sensitive substance in the range of wave-lengths which have been investigated (Lasareff, 1907). Experiments were therefore undertaken to determine the effectiveness of light of different wave-length on the photosensory responses of *Mya arenaria*.

3. The method used is in its essentials as follows. The reaction time of *Mya* to light varies inversely as the logarithm of the intensity (Hecht, 1920–21, *e*). The relation between these two variables may be found for light of any quality. When using monochromatic illumination, the curve expressing the relation between the intensity (I) and the reaction time (t) may be called an *It-isochrome*, in analogy to the curve at constant temperature called an *It-isotherm*. The experiments consist in mapping out the *It-isochromes* for the photic response of *Mya* to lights of different composition. The relations among the isochromes will give the relative effectiveness of the different lights.

The experiments were performed in the Marine Biological Laboratory at Woods Hole, Massachusetts, during the summer of 1920.

II.

1. Monochromatic light is secured by means of Wratten Light Filters Nos. 70 to 76 inclusive. These are made by the Eastman Kodak Company, and consist of specially stained gelatin films cemented between glass plates. The absorption spectra of these light filters

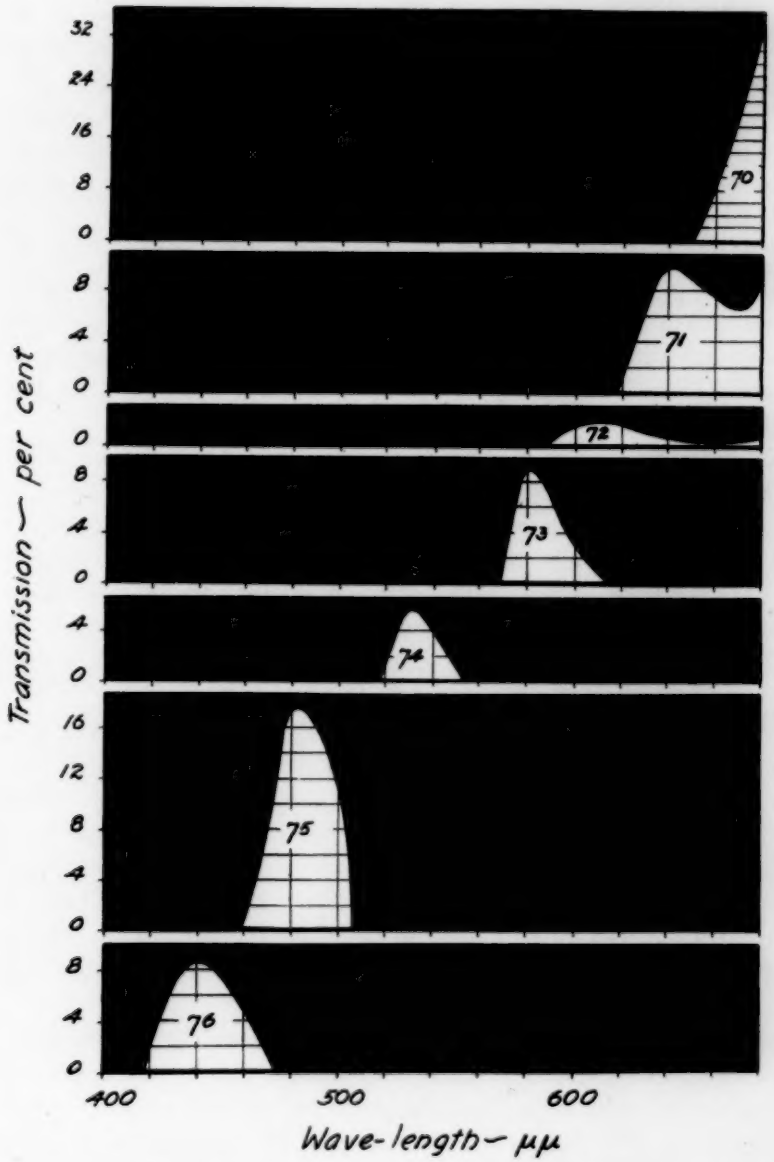

FIG. 1. Transmission spectra of the Wratten Light Filters used to secure monochromatic illumination.

have been carefully measured.[1] They are presented graphically in Fig. 1. The white spaces are the transmission areas for the different screens in terms of the per cent transmission of the incident light.

It is apparent that each screen transmits a rather narrow portion of the visible spectrum. In the significant part of the spectrum (λ = 400 to 600 $\mu\mu$) the screens transmit bands about 40 $\mu\mu$ wide; most of the light transmitted is confined to an even narrower band.

2. With these screens I used a 400 watt, concentrated-filament Mazda lamp, running on an ordinary lighting circuit of 115 volts. This source of light may be considered as a point source, and different relative intensities may be obtained by placing animals at different distances from the light, the intensities being computed on the inverse square law.

For ordinary sources of light the energy content of the spectrum varies with the wave-length. It is therefore important to know the exact values of the relative energy distribution of the light in order that the energy transmitted by the filters be known. The results of the determination of the energy distribution for the 400 watt lamp which I used in these experiments are shown in Fig. 2, which gives the relative energy content of the different wave-lengths of the visible spectrum.

From Fig. 2 we know the energy incident on the filters. Fig. 1 in turn tells us the portion of this incident energy which is transmitted by each of the filters. It is thus a simple computation to find out the energy content of the light transmitted by each filter in conjunction with the lamp. The transmission values in Fig. 1 and the energy values in Fig. 2 are both determined for bands 10 $\mu\mu$ wide. The total energy transmitted by any filter is therefore the sum of the energy transmitted by its constituent 10 $\mu\mu$ bands. These totals are given in Table I.

The Mazda lamp plus a screen may thus be considered as a source of energy which radiates a narrowly defined portion of the spectrum, and whose intensity is proportional to the energy content of the transmitted light. For convenience I have used for the relative intensity the same figures as those given in Table I for the transmitted energy.

[1] The measurements are given on p. 61 of a booklet—Wratten Light Filters—published by The Eastman Kodak Company.

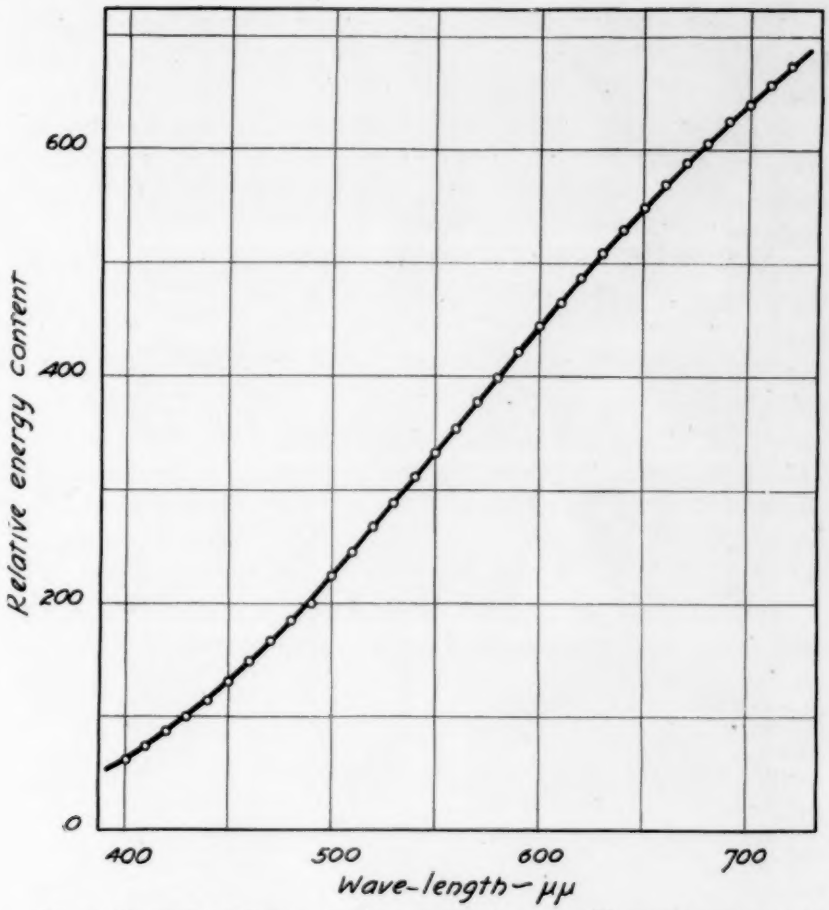

FIG. 2. Energy distribution in the spectrum of the 400 watt lamp used in conjunction with the light filters.

TABLE I.

Relative Energy Transmitted by Wratten Light Filters Plus a 400 Watt Lamp.

Filter No.	Energy.
70	366.8
71	285.3
72	34.8
73	88.6
74	36.8
75	106.9
76	38.1

The distribution of the energy transmitted by each screen is given in Fig. 3. For uniformity the total transmission is given the value of 100. It will be seen that the point of maximum energy transmission is occasionally shifted toward the red end as compared with the per cent transmission given in Fig. 1. This is, of course, because the energy content of the spectrum increases steadily toward the red end, as shown in Fig. 2.

3. In Figs. 1 and 3 the transmission of the filters is given only for the visible spectrum. All the filters transmit infra-red rays. It may, however, be said at once that rays beyond $\lambda = 680\ \mu\mu$ are ineffective as stimulating agents for *Mya*. I have determined this by subjecting animals to very intense illumination from which all the visible rays have been filtered out by means of Wratten Light Filter No. 87. This screen transmits the rays beyond $\lambda = 680\ \mu\mu$, and none below that wave-length. In no case did the animals respond to these infra-red rays. As a source of error, therefore, infra-red rays may be left out of consideration.

The screens do not transmit ultra-violet light. In addition, the light from the incandescent filament has to pass through several centimeters of glass before reaching the animal. Therefore, the ultra-violet rays are screened out in this way as well, and do not enter as a source of error.

The filters are quite photostable. But in order to avoid any possible bleaching effect, a shutter is placed between the light source and the filter, so that the filter is exposed to light only during the few seconds required to expose an animal and to measure its reaction time. Moreover, the light is turned off between exposures in order that as little heat be formed as possible. This is a necessary precaution, because a 400 watt lamp produces a tremendous amount of heat, which might otherwise pass even through the shutter and affect the gelatin between the glass plates of the filter.

From what has been said it is evident that these Wratten Light Filters furnish a simple method of procuring practically monochromatic light of known intensities, many times greater than can ordinarily be produced with the aid of a dispersion spectrum.

4. Further than the use of the screens, the experiments involve no new principle. A filter is placed in the path of the beam of light im-

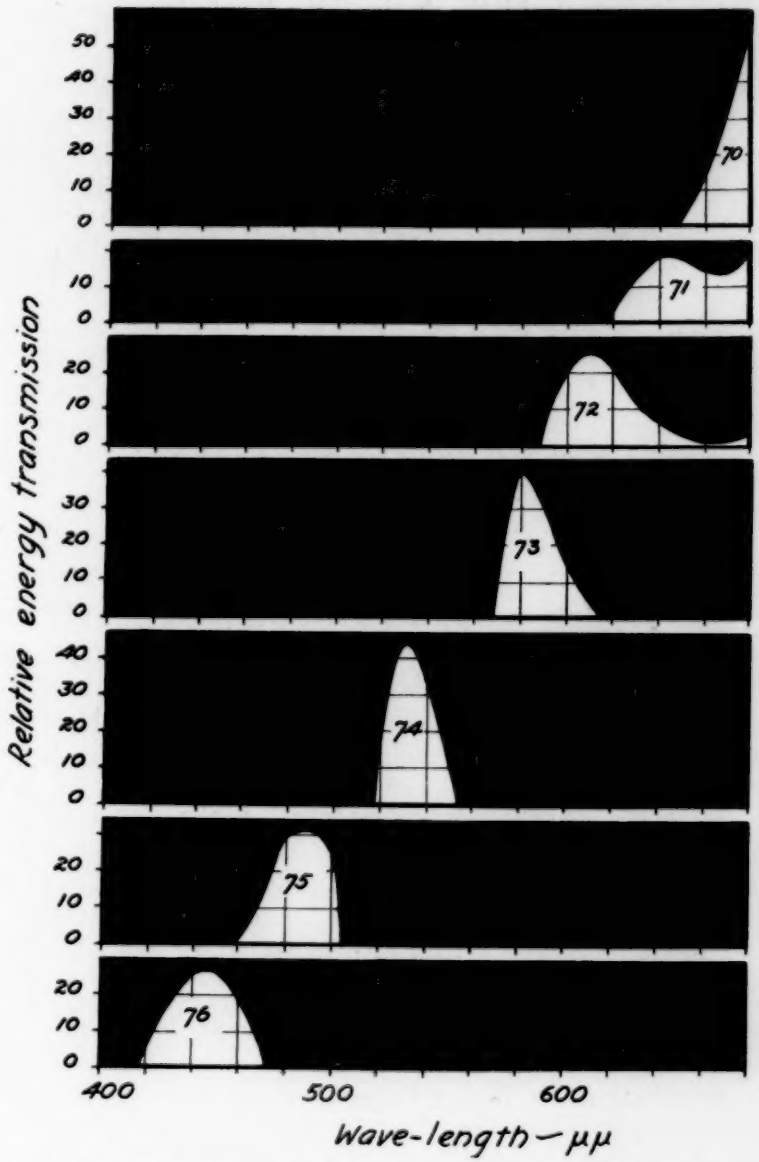

FIG. 3. Energy distribution of the light transmitted by the filters when used with the 400 watt lamp. The total energy for each filter is placed arbitrarily at 100. *Cf.* Table I.

mediately in contact with the shutter (for a description of the apparatus used, see Hecht, 1919–20, *c*). Several animals which have been thoroughly dark-adapted are then used. The reaction time of each animal is measured with a stop-watch. After a rest of 15 minutes in the dark, the animals are again tested, but at a distance from the light nearer or farther than before, depending on the magnitude of the previous reaction time. After another period in the dark, the reaction time is measured at still another intensity, and the process repeated until enough responses have been measured to cover a range between 2.0 and 4.0 seconds in the reaction time.

The curve giving the relation between the reaction time and the intensity is the *It*-isochrome for this filter. The entire procedure is then repeated with another filter, until all the filters have been tested. In this way a series of *It*-isochromes are mapped out for the seven portions of the visible spectrum shown in Fig. 3.

III.

1. Several preliminary experiments agreed in showing that the light coming through Filter 75 is the most effective portion of the spectrum. A final set of experiments was then arranged using nine dark-adapted animals. The results are given in Fig. 4. Each point is the average of nine determinations of the reaction time, one for each animal. The curves are the *It*-isochromes drawn smoothly through the points. An exception is the curve for Filter 76, which for obvious reasons is drawn parallel to the other six curves.

The range of intensities required to produce similar responses is very large; from 2 to 16,000 units. To present them in a single figure is manifestly impractical. Moreover, it has been shown that the reaction time is inversely proportional to the *logarithm* of the intensity. Consequently, the abscissæ of Fig. 4 are the logarithms of the intensities and not the intensities themselves. The use of such a logarithmic plot makes all the *It*-curves parallel to one another, and renders their comparison more simple than would otherwise be the case.

The *It*-isochromes of Fig. 4 show at once that the most effective portion of the spectrum is the light transmitted by Filter 75. This corresponds to a band whose maximum is at 490 $\mu\mu$, in the blue-green.

It requires the least amount of energy to produce a given photosensory effect.

2. It will be of significance to determine quantitatively the relative effectiveness of the different parts of the spectrum. The method used in this connection deserves a little consideration. It is required to find the relative effectiveness of a series of reagents on a given system. The common procedure of comparing the different effects produced by the same concentration of the reagents, though giving qualitative results, is utterly fallacious for quantitative purposes, except in rare instances such as when the effect produced is a linear function of the

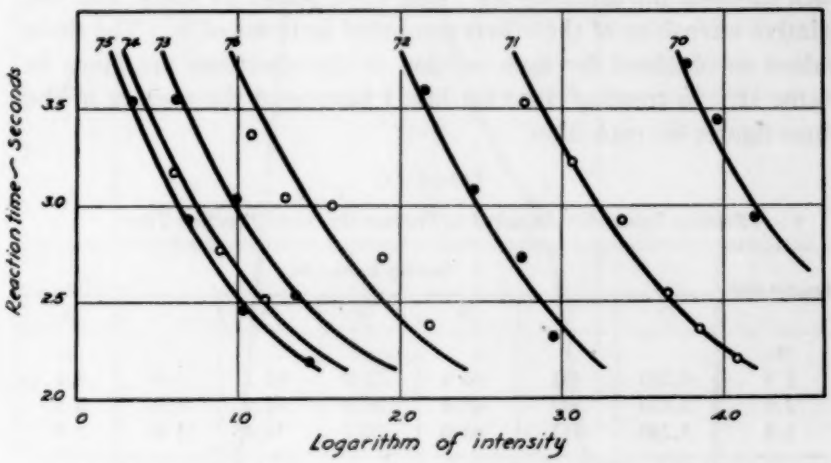

FIG. 4. *It*-isochromes for the responses of *Mya* to the different lights transmitted by the various filters.

concentration. A quantitatively correct evaluation requires the comparison of the concentrations of the different reagents which will produce the same effect.

I need not labor the point, because Brooks (1920) has presented it in some detail in its bearing on the theoretical interpretation of hemolysis data. Applied to our immediate problem, it follows that the important thing in working with spectral light is *not* to have a spectrum of *equal* energy distribution with which to determine the relative effects of different parts of it, but to have a spectrum of variable, *known* energy distribution, with which to determine the

energy required to produce the *same* sensory effect. The value of the latter method will become apparent when the attempt is made in the next section to interpret the experimental findings.

In the present instance the same sensory effect is represented by responses produced in the same reaction time. It is therefore required to measure the different intensities necessary to produce the same reaction time. This can be done by reading from the *It*-isochromes of Fig. 4 the intensities corresponding to definite values of the reaction time.

I have done this for three values; for 2.5, 3.0, and 3.5 seconds. In each instance the intensity for Filter 75 is placed at unity, and the relative intensities of the others computed in terms of it. The three values so obtained for each portion of the spectrum are given in Table II. In treating them further, I have used the average of the three figures for each filter.

TABLE II.

Relative Intensities Required to Produce the Same Reaction Time.

Reaction time.	Intensity for filter No.						
	70	71	72	73	74	75	76
Sec.							
2.5	4,030	452	66.9	22.8	13.2	1.0	5.1
3.0	3,650	392	65.4	22.0	12.9	1.0	6.5
3.5	3,240	312	63.0	20.7	12.6	1.0	5.3
Average...	3,640	385	65.1	21.8	12.9	1.0	5.6

3. The relative effectiveness of the different lights may be represented by the reciprocals of the relative intensities. Fig. 5 shows these values in graphic form. For convenience, and for another reason which will presently become apparent, the reciprocal of the maximum is made equal to 100, the other values being computed accordingly and employed as ordinates. The wave-length is used as abscissa. Each point has been plotted as a rectangle, whose center is the point of maximum energy transmission as given in Fig. 3, and whose horizontal dimension represents the points between which 75 per cent of the transmitted energy is confined. A smoothed curve through the points shows strikingly that the most effective portion

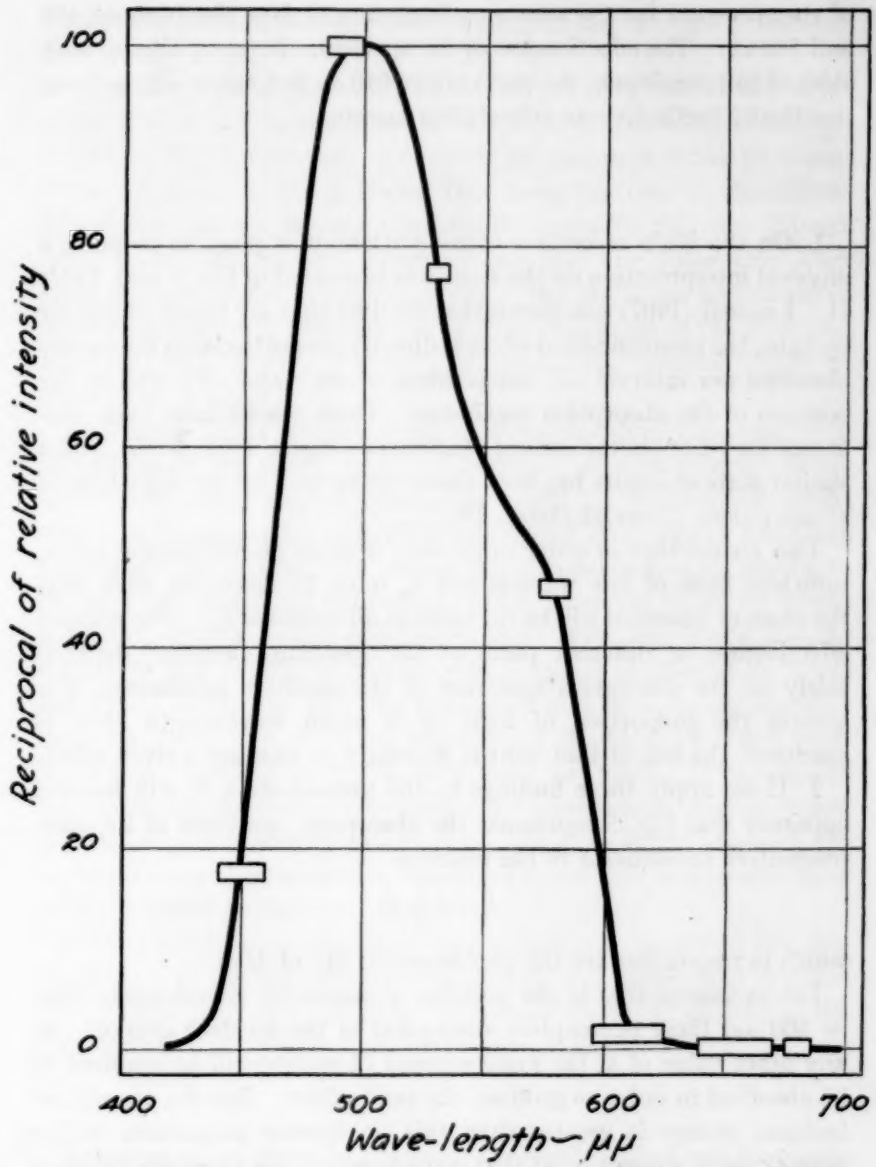

FIG. 5. Relative effectiveness of the spectrum in sensory stimulation. The curve also represents the absorption spectrum of the photosensitive substance of *Mya*. In the latter case the ordinates represent per cent of absorption by the sensitive substance.

of the spectrum for the sensory stimulation of *Mya* lies between 490 and 510 $\mu\mu$. The effectiveness of the spectrum drops rapidly on both sides of this maximum, the parts above 610 $\mu\mu$ and below 420 $\mu\mu$ being practically ineffective as stimulating agents.

IV.

1. On the basis of certain considerations it is possible to place a physical interpretation on the results as presented in Fig. 5 and Table II. Lasareff (1907) has shown that for dyes that are bleached rapidly by light, the photochemical effect is directly proportional to the energy absorbed per interval $\Delta\lambda$, independent of the value of λ and of the position of the absorption maximum. These results have been confirmed on other photochemical reactions (Weigert, 1911, p. 90); and a similar state of affairs has been shown to be true for the bleaching of visual purple (Trendelenburg, 1911).

This means that in order to produce a given photochemical effect, sufficient light of any wave-length λ_n must be delivered, such that the amount absorbed will be the same at all values of λ_n. The relative effectiveness of different parts of the spectrum therefore depends solely on the absorption spectrum of the sensitive substance. The greater the proportion of light of a given wave-length that is absorbed, the less of that light is necessary to produce a given effect.

2. If we apply these findings to the present data, it will become apparent that Fig. 5 represents the absorption spectrum of the photosensitive substance *S* in the reaction

$$S \rightarrow P + A$$

which is responsible for the photic sensitivity of *Mya*.

Let us assume that in the position of maximum effectiveness (λ_{max} = 500 $\mu\mu$) there is complete absorption of the incident energy. At any other value of λ_n the same amount of energy will be required to be absorbed in order to produce the same effect. But the amount of incident energy is greater than this, in inverse proportion to the magnitude of absorption at that wave-length. We know the incident energy from Figs. 4 and 5 and Table II. The absorbed energy is equal to the amount required at the maximum effectiveness (λ =

500 $\mu\mu$). At any other wave-length λ_n, then, the per cent of absorption will be given by the ratio of the energy at λ_{max} divided by the incident energy at λ_n.

The absorption at λ_{max} will then be 100 per cent, and at any other value of λ_n it will be equal to the reciprocal of the incident energy. Therefore Fig. 5 represents the absorption spectrum of the photosensitive substance *S*, the ordinates now being per cent of absorption. Absorption spectra are not infrequently given in this way (Henri, 1919, p. 42, 65). I shall therefore not give the absorption spectrum in terms of the absorption index. The latter can be calculated according to certain assumptions as to the thickness of the absorbing layer etc., (*Cf.* Bovie, 1918–19), in themselves, however, only of speculative interest.

3. Because of its interpretation as the absorption spectrum of *S*, the shape of the curve in Fig. 5 becomes of significance. Many known substances possess absorption bands in position and extent similar to that shown here (*Cf.* Uhler and Wood, 1907, Fig. 17 and those following). As a rule such curves are symmetrical with regard to the point of maximum absorption. This, however, is by no means universal, because many substances show skew absorption spectra similar to that in Fig. 5; for example, uranine, as studied by Uhler and Wood (1907, Fig. 15). In such cases it is usual to assume that there are really two, or more, vibrators in the molecule, their combined effect being given by the total curve as found. If we suppose that in the photosensitive substance *S* there are present two vibrators, one whose period corresponds to 500 $\mu\mu$ and the other to about 570 $\mu\mu$, each giving a symmetrical resonance curve, the compound curve of Fig. 5 would be their resultant.

Leaving aside these speculative matters, it may be noted that the appearance of the absorption curve, though not particularly distinctive, is sufficiently so to serve as a corroboration of the identity of the photosensitive substance *S* in future experimentation.

V.

In recent years there has appeared a number of careful measurements of the most effective portion of the spectrum for the stimulation of different organisms (Laurens and Hooker, 1920; Loeb and

Wasteneys, 1916; Mast, 1917). Although for many animals this point lies in the yellow-green, and for many plants in the blue-green, there is an increasing number of organisms which do not conform to this classification. *Mya arenaria* clearly belongs in this non-conforming category, because it is an animal for which the maximum effectiveness is in the blue-green.

The particular significance of the above attempts at classification is due to Hess (1910). As the result of experiments with a number of animals, Hess has come to the conclusion that "the curves of the relative stimulating values of different homogeneous lights coincide, approximately or exactly, with the luminosity curve of the totally color blind human eye" (Hess, 1910, p. 362). From which he proceeds to draw certain conclusions with regard to the nature of the responses of animals to light. Loeb and Wasteneys (1916) have shown that these conclusions not only fail to agree with the facts of other investigators, but also that the process of arriving at them involves a decided logical fallacy. If after this any additional evidence were needed, the results with *Mya* certainly show that Hess' original statement is not generally true. The curve of effectiveness of the spectrum for *Mya* possesses nothing in common with the luminosity curve for the color blind or the dark-adapted human eye.

It may be added that Hess' method of experimentation is open to criticisms which invalidate his conclusions. Working with certain clams whose photic sensitivity is similar to that of *Mya*, he employs criteria of the effectiveness of light which cannot be considered quantitative. He notes the length of the extended siphon, or the extent of its retraction as the animal is moved along the projected spectrum. My experience with *Mya* has been that these are variable characteristics, even when care is taken to insure uniformity otherwise. In Hess' experiments no values are given for the energy content of the spectrum; no time is allowed for the recovery of an animal in the dark between stimulations; no measurements are made of the time of exposure; no record is made of the reaction time. In fact, few measurements of any kind are given. Even the "curves" on which the comparison with the human eye rests fail to appear in the article (Hess, 1910). Thus, even if Hess' logic were correct, his experimental findings are open to grave doubt; and the conclusions drawn from them are therefore doubly invalid.

The results with spectral lights therefore sum up to this. With *Mya* the effectiveness of the spectrum depends exclusively on the absorption spectrum of the photosensitive substance in the sense organ. The different positions of the maximum effectiveness for various organisms merely show that the sensitive substances concerned are different entities, each possessing its own absorption spectrum.

SUMMARY.

1. Following the description of a simple method of securing high intensities of monochromatic illumination, it is shown that the most effective portion of the spectrum for the stimulation of *Mya* is near $\lambda = 500\ \mu\mu$.

2. The quantitative data secured is interpreted in terms of certain photochemical findings, and as a result the absorption spectrum of the photosensitive substance of *Mya* is tentatively mapped out.

Through the courtesy of Doctor M. Luckiesch of the Nela Research Laboratory, Mr. W. E. Forsythe and Mr. F. E. Cady determined the energy distribution for the 400 watt lamp which I used in these experiments. I take pleasure in thanking them for their help in this connection.

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ION SERIES AND THE PHYSICAL PROPERTIES OF PROTEINS.

III. The Action of Salts in Low Concentration.

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(Received for publication, November 9, 1920.)

I. The Difference in the Effect of Acids, Alkalies, and Salts on Proteins.

The data concerning electrolytic dissociation and the behavior of electrolytes in general suggest that it is well to discriminate between solutions of electrolytes in low and in high concentrations. While no sharp line of demarcation can be drawn it will suffice for the problem we are discussing to designate a concentration of electrolytes below M/16 as low and those near the solubility limit of one of the components as high. In this paper we intend to deal with solutions of low concentrations; *i.e.*, M/16 or less.

It has been noticed by a number of authors that the influence of neutral salts on the physical properties of proteins differs from that of acids and bases; and various attempts have been made to find an expression for this difference. Pauli[1] states that while acids and alkalies form salts with proteins, neutral salts form "adsorption compounds" with "electrically neutral," *i.e.* non-ionized, protein molecules, both ions of the salt being simultaneously adsorbed by the protein molecule. This idea is no longer tenable for salt solutions of low concentration since the writer has shown through his experiments with powdered gelatin that only one (or practically only one) of the two ions of a neutral salt can combine at one time with a protein. At the isoelectric point, *i.e.* at pH 4.7, gelatin can combine with neither ion of a neutral salt; at a pH > 4.7 only the metal ion of the neutral salt can combine with the gelatin, forming metal gelatinate;

[1] Pauli, W., *Fortschr. naturwiss. Forschung*, 1912, iv, 223.

at a pH < 4.7 only the anion of the neutral salt is capable of combining with the protein, forming gelatin-acid salts.[2]

Lillie has made the statement that while acids and alkalies increase, salts depress the osmotic pressure of gelatin.[3] This statement, while it was the expression of facts actually observed by Lillie, is no longer tenable owing to the fact that the influence of the hydrogen ion concentration of the gelatin solution was not taken into consideration. If we add acid to a gelatin-acid solution of a pH of 3.0 or below, the effect is practically the same as when we add a neutral salt, namely a diminution of the osmotic pressure of the solution; and when we add alkali, *e.g.* KOH, to a solution of a metal gelatinate of pH 11.0 or above, the effect is also a similar depression of the osmotic pressure as that caused by the addition of KCl. We also get a depression when we add some acid to a solution of metal gelatinate or when we add some alkali to gelatin-acid salts; since in both cases the gelatin is brought nearer to the isoelectric point.

It is also incorrect to speak of an antagonism between the effects of acids and salts, since the facts mentioned show that there is also an antagonism between little and much acid; thus if the pH of a gelatin-acid salt is 3.0 a further addition of acid depresses the osmotic pressure or viscosity. The question then arises, what is the correct expression of the facts in the case?

An analogy with another field of phenomena may be of service. The writer has recently published a series of articles on the influence of electrolytes on the rate of diffusion of water through collodion membranes, which have shown that water diffuses through such a membrane as if the particles of water were positively charged. When pure water is separated from a solution of an electrolyte of not too high a concentration, the positively charged particles of water diffuse through the collodion membrane into the solution as if they were attracted by the anion and repelled by the cation with a force increasing with the valency of the ion. In this case the oppositely charged ions of an electrolyte influence the rate of diffusion of water through the membrane in an opposite sense.

[2] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 237; *Science*, 1920, lii, 449.
[3] Lillie, R. S., *Am. J. Physiol.*, 1907–08, xx, 127.

The second fact which was brought out was that the relative strength of the apparent attractive and repelling action of the oppositely charged ions of an electrolyte upon the electrically charged particles of water varies with the concentration of the electrolyte in the solution. In the lower concentrations of the electrolyte the attractive action of the anion upon the positively charged particles of water increases more rapidly with increasing concentration of the salt than the repelling action of the cation; while when a certain concentration is reached the repelling action of the cation upon the positively charged particles of water increases more rapidly with a further increase in the concentration of the salt than the action of the anion.[4]

These facts offer an analogy which is helpful in understanding the difference in the action of acids and alkalies on the one hand, and of neutral salts on the other upon the physical properties of proteins.

When acids or alkalies are added to isoelectric gelatin both ions of the acid or alkali influence the physical properties of proteins but in an opposite direction. When we add acid to isoelectric protein the hydrogen ions increase, the anions depress the osmotic pressure and viscosity of the protein solution (and this depressing action increases with the valency of the anion of the acid). As long as little acid is added to isoelectric protein the augmenting action of the hydrogen ion on these properties increases more rapidly with increasing concentration of the acid than the depressing action of the anion; while when the pH of the solution falls below 3.3 or 3.0 the reverse is the case. This causes the drop in the curves for osmotic pressure, viscosity, and swelling below a pH of 3.0.

When we add alkali to isoelectric protein the OH ions (or the diminution of the concentration of hydrogen ions) increase the osmotic pressure, viscosity, etc., of the solution of metal proteinate while the cation of the alkali depresses these properties with a force increasing with the valency of the cation. In the lowest concentrations of the alkali added the augmenting action of the OH ion on the physical properties of the metal proteinate increases more rapidly with the concentration than the depressing effects of the cation of the alkali; while in higher concentrations, *i.e.* as soon as the pH becomes about 10.0 or 11.0, the reverse is the case.[5]

[4] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 173.
[5] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 85, 247.

When, however, neutral salts are added to protein solutions we no longer notice an opposite effect of the oppositely charged ions. When neutral salts are added to isoelectric gelatin no effect is noticed as long as the concentration of salt does not reach the value required for precipitation. When neutral salt is added to a protein solution on either side of its isoelectric point only a depressing action of that ion which has the opposite sign of charge as the protein ion is observed. No augmenting action of the ion with the same sign of charge as the protein is noticeable. Thus if we add $CaCl_2$ or Na_2SO_4 to a solution of gelatin chloride or gelatin nitrate we observe only a depressing effect of the Cl or SO_4 ion but no augmenting effect of the Ca or Na ion; while when we add these salts to a solution of a metal gelatinate we observe only a depressing effect of the Ca or Na ion but no augmenting effect of the anion. We shall first show that this is the correct expression for the difference in the action of acids and bases on the viscosity of proteins on the one hand and of neutral salts on the other.

A 2 per cent solution of isoelectric gelatin is prepared and brought to a pH of 4.0. The solution is made 1 per cent in regard to the originally isoelectric gelatin by adding to 50 cc. of the 2 per cent solution either 50 cc. of H_2O or of a salt solution, *e.g.* NaCl, of different molecular concentration, from M/8,192 to 1 M, taking care that the hydrogen ion concentration remains the same. We determine the viscosity (*i.e.* the time of outflow through a viscometer) in the way described in a preceding publication and plot the reciprocal of time of outflow (counting that of water as 1) as ordinates over the pH as abscissæ (lower curve, Fig. 1). For the sake of briefness we beg leave to designate this value as specific viscosity.[5] The addition of the NaCl causes only a drop, and no rise in the curve.

If, however, we mix the 2 per cent gelatin solution of pH 4.0 with various concentrations of HCl (upper curve, Fig. 1) instead of with NaCl we do not notice a drop but at first a rise followed by a drop when the concentration of the Cl ion is a little above N/1,000. In Fig. 1 the drop appears at a concentration of about N/256 HCl, but the reader must remember that on account of the fact that part of the acid combined with the gelatin the pH of the solution was about 3.0. In other words, while the addition of H ions increases the vis-

cosity of a solution of gelatin chloride of pH 4.0, the addition of Na ion does not have such an effect, but the Cl ion depresses the viscosity in both cases, no matter whether NaCl or HCl is added to the gelatin

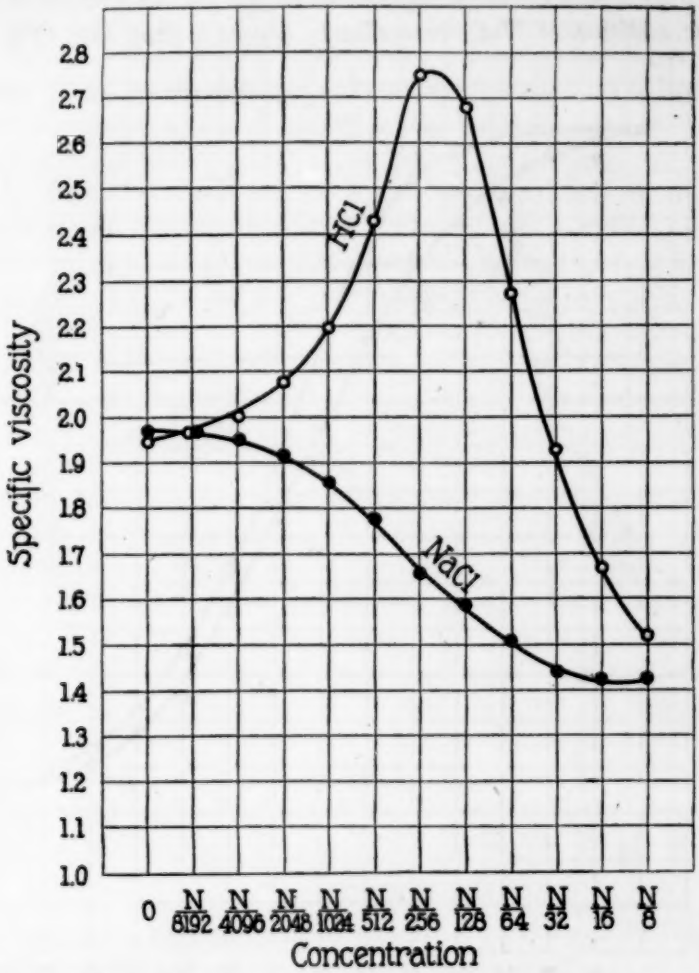

FIG. 1. Difference in the effect of different concentrations of NaCl and of HCl on the specific viscosity of a 1 per cent solution of gelatin chloride of pH 4.0. In the case of NaCl we observe only the depressing effect of the Cl ion; in the case of HCl we notice an augmenting effect of the H ion and a depressing effect of the Cl ion, the latter prevailing as soon as the concentration of acid added is > N/256.

solution; and the depressing action of the Cl ion increases with its concentration. Moreover, the increase of the viscosity by the H ions stops as soon as the pH of the solution reaches about 3.0.

When we repeat the same experiment with a gelatin solution of pH 3.0, the addition of NaCl immediately causes a drop also (Fig. 2)

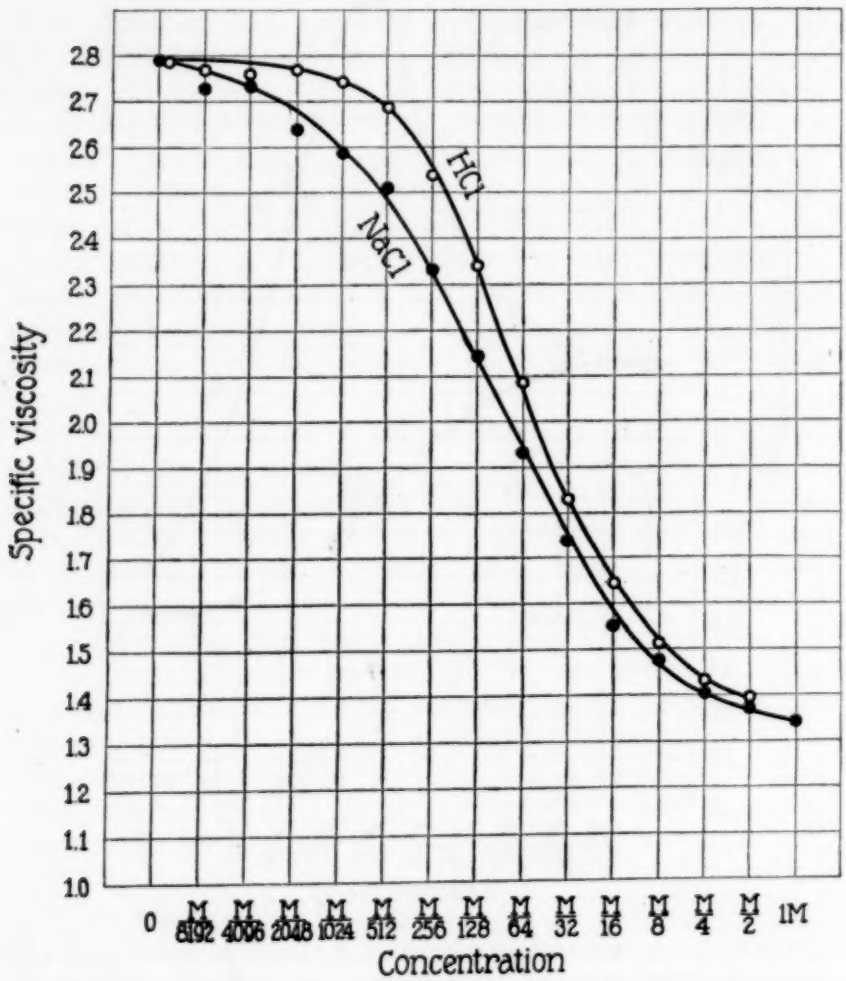

FIG. 2. The specific viscosity of 1 per cent solution of gelatin chloride of pH 3.0 is depressed almost equally by the Cl ion of HCl as of NaCl. The augmenting effect of the H ion in the case of HCl is no longer noticeable.

while the addition of HCl no longer causes a rise but the drop commences a little later than in the case of NaCl.

When, however, we make the same experiment with a gelatin solution of pH 2.5 (Fig. 3), we notice an immediate drop upon the

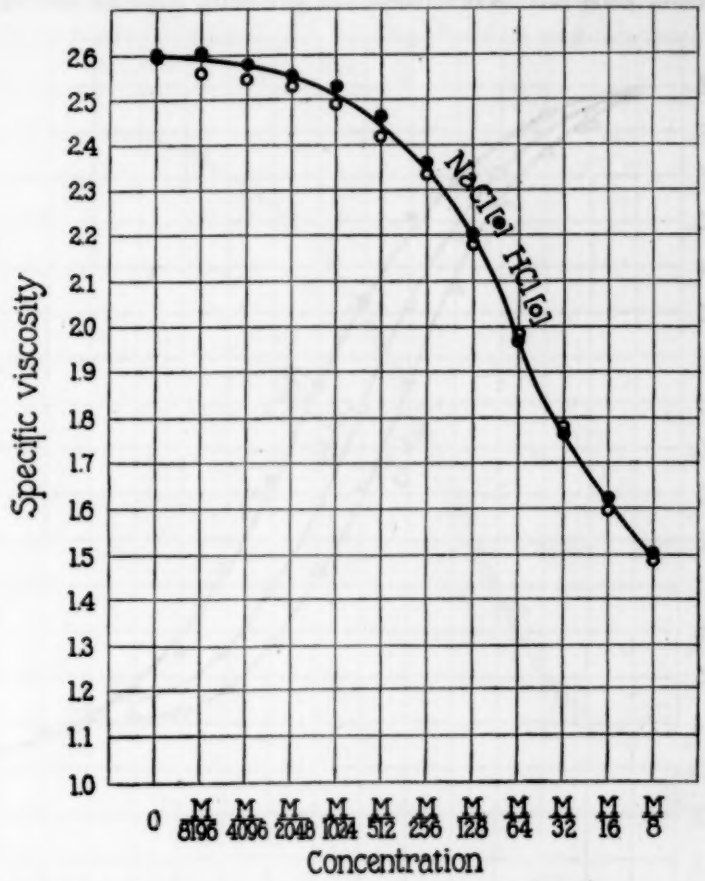

FIG. 3. When the gelatin solution has a pH of 2.5, HCl and NaCl depress the specific viscosity of the gelatin solution to the same degree.

addition of HCl as well as in the case of the addition of NaCl, and the curve for HCl coincides practically with that for NaCl, as our theory demands.

That the depression of the viscosity of gelatin chloride due to the presence of a salt is exclusively determined by the anion of the salt

and that the cation has no augmenting effect is shown in Fig. 4, where the influence of NaCl, $CaCl_2$, and $LaCl_3$ upon the viscosity of gelatin of pH 3.0 is represented. 50 cc. of a 2 per cent solution of gelatin chloride of pH 3.0 were added to 50 cc.of a solution of different concentrations of each salt as described, the pH being kept at 3.0. It is

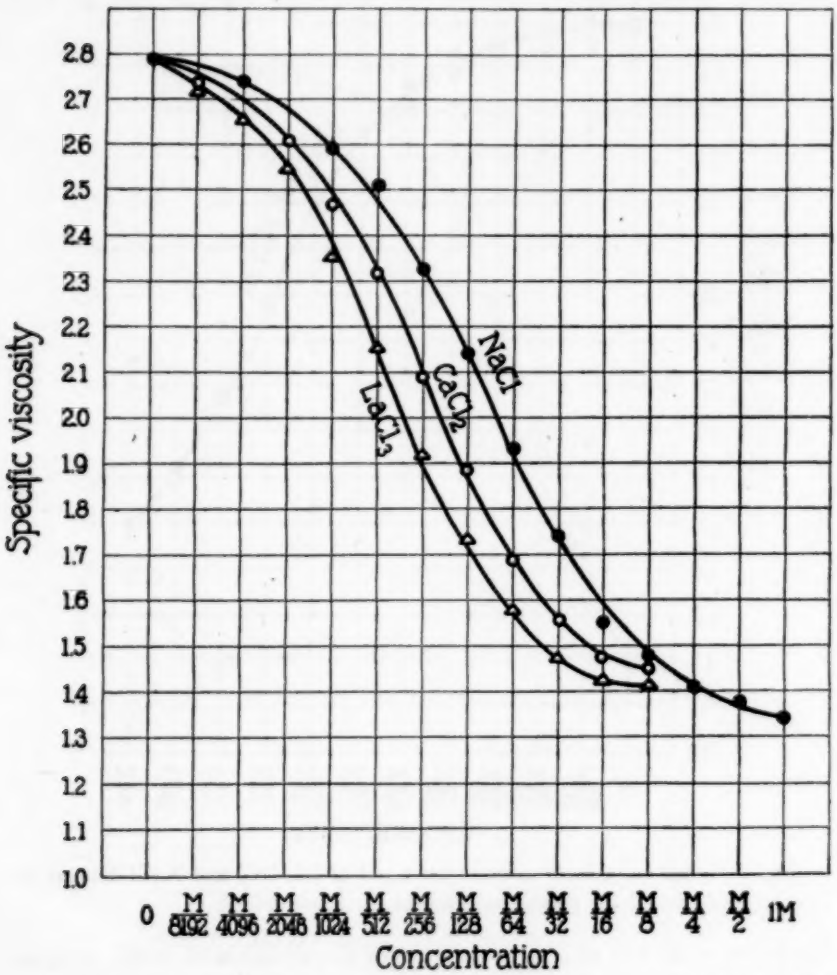

FIG. 4. The depressing effect of equal molecular concentrations of NaCl, $CaCl_2$, and $LaCl_3$ on the specific viscosity of 1 per cent gelatin chloride solution of pH 3.0 is in exact proportion to the concentration of the Cl ions in the solution; *i.e.*, as 1:2:3.

obvious from Fig. 4 that the molecular concentrations of NaCl, $CaCl_2$, and $LaCl_3$, which depress the viscosity to the same level are approximately in the ratio of 3:2:1. Thus when the effect of NaCl and $CaCl_2$ is plotted over the same concentration of the Cl ions the curves for the salts become identical (Fig. 5), and the same would be practically

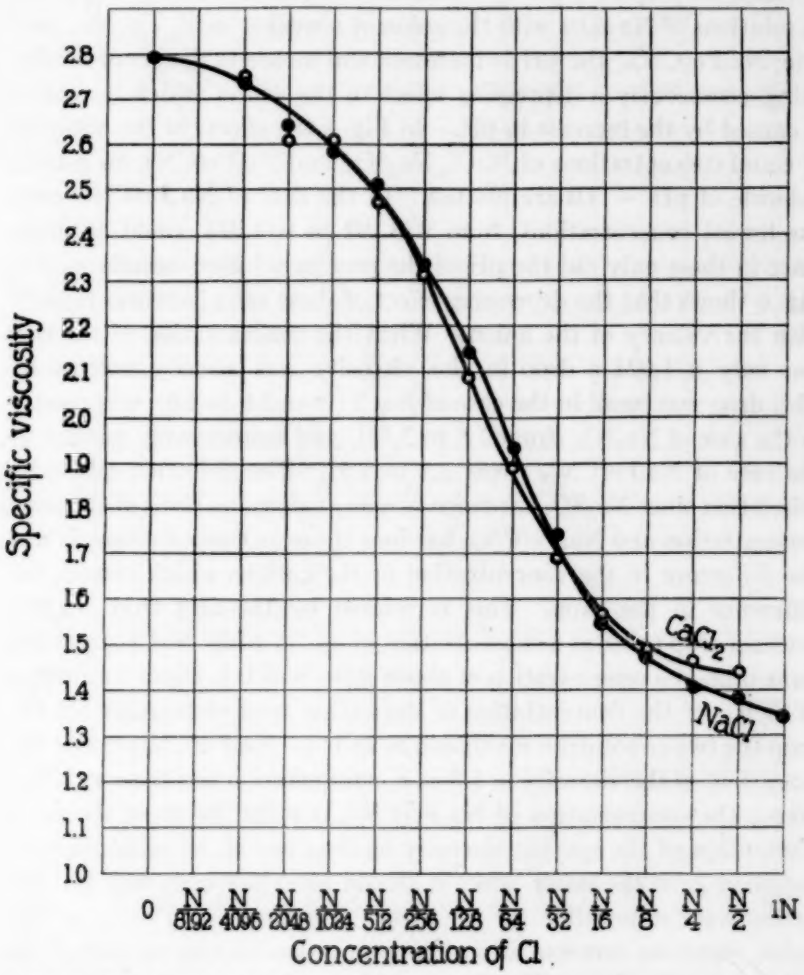

FIG. 5. Showing that NaCl and $CaCl_2$ have the same depressing effect on the viscosity of gelatin chloride of pH = 3.0 when the concentration of Cl ions is the same.

true for the $LaCl_3$ curve. From this it follows that the depressing effect of these three salts on gelatin chloride is practically exclusively a function of the concentration of the Cl ion, while no effect of the cation seems to be noticeable. In all these cases the pH of the gelatin solution was not altered by the addition of the salt.

When we prepare 1 per cent solutions of gelatin chloride of pH 3.0 in solutions of Na salts with the anion of a weaker acid, *e.g.* Na_2 oxalate, $Na_4Fe(CN)_6$, the pH is increased and we are in danger of attributing erroneously a depressing effect to the anion which in reality is caused by the increase in pH. In Fig. 6 the effects of the addition of equal concentrations of NaCl, Na_2SO_4, and $Na_4Fe(CN)_6$ on gelatin chloride of pH = 3.0 are plotted. In the case of $Na_4Fe(CN)_6$ only the lowest concentrations, from M/8,192 to M/1,024, could be used, since in these only did the pH of the protein solution remain = 3.0. Fig. 6 shows that the depressing effect of these salts increases rapidly with the valency of the anion. When the concentration of the salt was only M/1,024 a drop in the viscosity was already noticeable. This drop was small in the case of NaCl (from 2.8 to 2.6), was greater in the case of Na_2SO_4 (from 2.8 to 2.35), and considerably greater in the case of $Na_4Fe(CN)_6$ (from 2.8 to 1.5). The objection might be raised that since Na_2SO_4 has twice as many cations as NaCl of the same concentration and $Na_4Fe(CN)_6$ has four times as many cations, it was the difference in the concentration of the cations which caused the difference in the drop. This is refuted by the fact that Na_2SO_4 causes a drop to 1.8 at a concentration of M/256 while NaCl causes the same drop at a concentration of above M/64 which is about four times as high. If the concentration of the cation were responsible for the drop the two concentrations should be as 1:2. $Na_4Fe(CN)_6$ causes the same drop of the viscosity to 1.8 at a concentration less than M/1,024. Hence the concentration of $Na_4Fe(CN)_6$ required to cause the same diminution of the specific viscosity as that caused by M/64 NaCl is less than $\frac{1}{16}$ of the latter, while it should be at the least only $\frac{1}{4}$ if the cation were responsible for the drop. The depressing effect of the anion seems to increase almost in proportion to the square of its valency, as the Hardy-Whetham rule demands.

We have selected viscosity experiments, but experiments on osmotic pressure and on swelling lead to the same formulation of the difference in the effect of acids and salts.

What has been shown for the effect of acids on the physical properties of proteins can also be shown for the influence of alkalies. Thus the addition of KOH to Na gelatinate of pH 12.0 depressed the

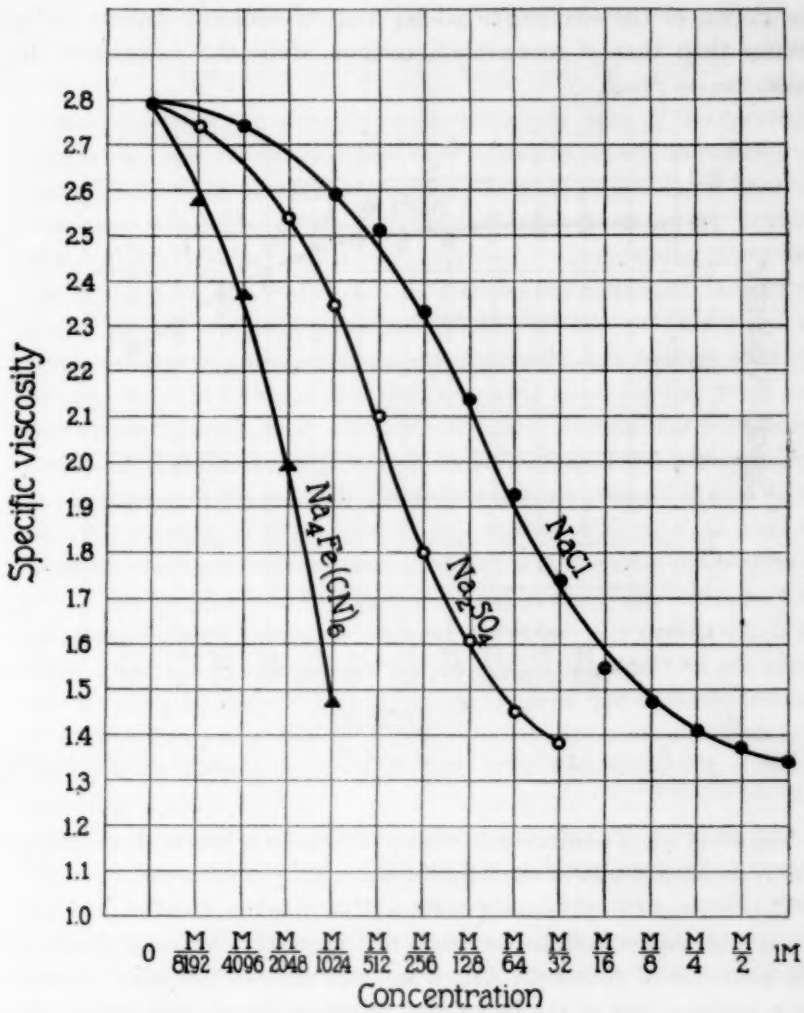

FIG. 6. The relative depressing effect of equal molecular concentrations of NaCl, Na_2SO_4, and $Na_4Fe(CN)_6$ on the specific viscosity of a gelatin chloride solution of pH 3.0 is approximately as 1:4:16.

viscosity in the same way as the addition of KCl (Fig. 7); while the addition of KOH to Na gelatinate of pH 4.8 to 8.0 increases the viscosity and the addition of KCl to Na gelatinate always depresses the viscosity. The depressing effect of salts on metal gelatinate is due to the cation of the electrolyte added, that of bivalent cations being greater than that of monovalent cations, while the valency of the anion has no effect.

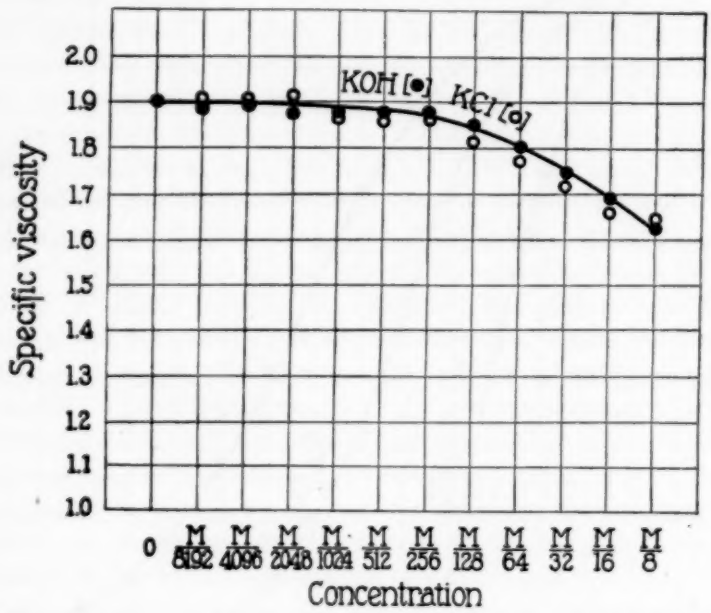

FIG. 7. The depressing effect of KOH and KCl on Na gelatinate of pH 12.0 is practically the same.

The addition of neutral salt to isoelectric gelatin leaves the viscosity and osmotic pressure of the solution practically unchanged.

The cause of this difference in the action of acids, alkalies, and salts is easy to understand on the basis of the purely chemical theory of the behavior of proteins. When we add acid to isoelectric gelatin we transform part of the latter into gelatin-acid salt and the proportion of gelatin-acid salt formed increases with the amount of the acid added. Near the isoelectric point the amount of gelatin-acid salt formed increases very rapidly with the addition of acid, but when

the pH approaches 3.0 the addition of the same amount of acid which near the isoelectric point caused a considerable change now causes only a slight change, while when the pH falls below 3.0 the depressing influence of the anion continues to increase with increasing concentration of the electrolyte.

II. Ion Series and the Action of Salts on Proteins.

We have shown in preceding papers that, as long as the concentration of the electrolyte is not too high, only the sign of the charge and the valency of an ion influence such physical properties as swelling, viscosity, solubility in alcohol, and osmotic pressure of proteins; while all the different ions of the same sign of charge and valency have either the same effect or if there is a difference in effect it is too small to be noticed with our present methods of work.[5] This proof was furnished for the action of acids and alkalies on the physical properties of proteins just mentioned. We now wish to make the proof complete by considering also the action of salts. To do this we are compelled to compare the relative depressing action of low but equal concentrations of different salts upon the physical properties of a gelatin salt, for example gelatin chloride of a definite pH; *e.g.*, 3.0. As can be easily surmised the addition of a salt will in many cases alter the pH of the solution and this alteration will be larger in the case of certain salts, *e.g.* Na acetate, than in the case of others, *e.g.* NaCl. Unless we take into consideration these variations in the pH caused by the addition of salts we shall be in danger of erroneously ascribing the influence of a variation in the hydrogen ion concentration to an influence of the nature of the anion. The Hofmeister ion series are due to this error.

The method of our experiments was as follows. 50 cc. of a 2 per cent solution of originally isoelectric gelatin were brought to a pH of 3.0 by the addition of HCl. To this were added 50 cc. of H_2O or of a salt solution of different molecular concentration, and the viscosity of this mixture was measured using those precautions which were described in a preceding paper.

Fig. 8 gives the curves representing the depression of the specific viscosity of a gelatin chloride solution of pH 3.0 by different concentrations of salts with monovalent anion; namely, NaCl, NaH_2PO_4,

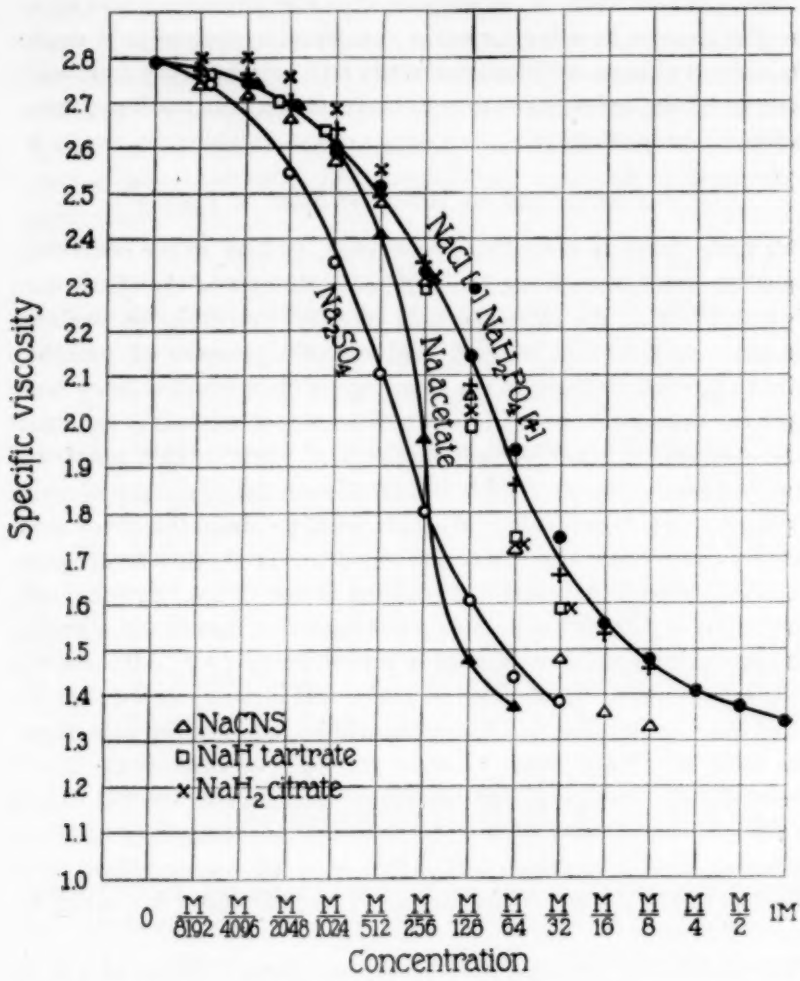

FIG. 8. The depressing effect of different salts with monovalent anion (NaCl, NaH_2PO_4, NaCNS, NaH tartrate, and NaH_2 citrate) on the specific viscosity of 1 per cent solution of gelatin chloride of pH 3.0. The effects of NaCl and NaH_2PO_4 are identical since the pH is not altered by the addition of these salts. The depression in the values for the specific viscosity is greater in the case of Na acetate than in the case of NaCl for the reason that the Na acetate raises the pH of the gelatin solution.

NaCNS, NaH tartrate, NaH_2 citrate, and Na acetate. The curve for Na_2SO_4 is added for comparison. The monosodium salts of weak dibasic and tribasic acids dissociate electrolytically into a Na ion and a monovalent anion, H_2PO_4, H tartrate, H_2 citrate, etc. All the salts mentioned in Fig. 8 are therefore salts with monovalent anion with the exception of Na_2SO_4. Our valency rule demands that the relative depressing effect of these salts (with the exception of Na_2SO_4) should be the same and that deviations from this rule should find their explanation in corresponding deviations of the pH due to the influence of certain of the salts. We will first consider this latter influence as given in Table I, which shows the results of the measurements of pH

TABLE I.

Changes in pH of 1 Per Cent Gelatin Chloride of pH = 3.0 upon Addition of Various Concentrations of Salts.

	Molecular concentrations of salts used.												
	0	M/8,192	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8	M/4
NaCl	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Na_2SO_4	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.05	3.1	3.2	3.3	3.35
NaH_2PO_4	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.1	3.2	3.3	3.4	3.45	3.5
NaCNS	3.0	3.0	3.0	3.0	3.0	3.0	3.1	3.2	3.3	3.6	3.9	4.2	4.4
NaH tartrate	3.0	3.0	3.0	3.0	3.0	3.0	3.1	3.3	3.45	3.5	3.55		
NaH_2 citrate	3.0	3.0	3.0	3.0	3.0	3.1	3.2	3.4	3.6	3.7	3.75		
Na acetate	3.0	3.0	3.0	3.05	3.1	3.3	3.7	4.3	4.6				

in these different gelatin solutions after the addition of salts. The original gelatin chloride solution had a pH of about 3.0 and this was not altered by the addition of NaCl and only slightly by the addition of NaH_2PO_4 in concentrations below M/16. According to the valency rule the curves for the depressing effect of NaCl and NaH_2PO_4 should be almost identical and Fig. 8 shows that this is the case.

Table I shows that NaCNS, monosodium tartrate, and monosodium citrate raise the pH of the solution as soon as the concentration reaches M/128 or more. If we consider this effect, we must expect to find that the drop in the curves for NaCNS, monosodium citrate, and monosodium tartrate is a little steeper in concentrations of M/128 and

above than the curve for the depressing effect of NaCl. Fig. 8 shows that the curves for the depressing effect of these three salts are slightly lower than the curve for NaCl or NaH_2PO_4. The greatest apparent deviation from the valency rule occurs in the curve for Na acetate whose depressing effect is of the order of that of Na_2SO_4.

In the colloidal literature it is always stated that Na acetate acts like Na_2SO_4 and this is interpreted to mean that the acetate anion acts like the bivalent SO_4 anion and not like the monovalent Cl or NO_3 anion. Table I shows that Na acetate also depresses the hydrogen ion concentration more than NaCl or NaH_2PO_4; M/64 Na acetate brings the gelatin solution practically to the isoelectric point, and at the isoelectric point the viscosity of gelatin solution is a minimum. This lowering of the hydrogen ion concentration (and not the alleged influence of the acetate anion) explains the excessive depressing effect of Na acetate. That this interpretation is correct can be proved in the following way. We prepare 1 per cent solutions of gelatin acetate of pH 3.3 and gelatin chloride also of pH 3.3. The specific viscosity of these two solutions was practically the same (both were 1 per cent solutions in regard to originally isoelectric gelatin). The solution of gelatin acetate of pH 3.3 was made up in various concentrations of Na acetate of pH 3.3. The Na acetate solution of pH 3.3 was obtained by dissolving M/16 Na acetate in $1\frac{1}{2}$ M acetic acid and the various degrees of dilution of this M/16 Na acetate solution of pH 3.3 were brought about by dilution with pure acetic acid of pH 3.3. The non-dissociated molecules of acetic acid have no more depressing influence on the physical properties of proteins than have the molecules of any non-electrolyte. Fig. 9 gives the curve representing the depressing effect of Na acetate on gelatin acetate of pH 3.3 when the pH is kept constant.

The gelatin chloride solution of pH 3.3 was made up in different concentrations of NaCl and the depressing effect of NaCl on the viscosity of gelatin chloride is also plotted in Fig. 9. It is obvious from Fig. 9 that the depressing effect of Na acetate and NaCl are identical when the pH is kept constant and identical in both cases.

The same fact was confirmed in a somewhat different way. A 2 per cent solution of gelatin chloride of pH 3.0 was made up in various concentrations of Na acetate also of pH 3.0. In order to prepare Na

acetate solutions of pH 3.0 M/4 Na acetate was dissolved in M/4 HCl and the various dilutions required for the experiment were obtained by diluting the mixture of equal parts of M/4 HCl and M/4 Na acetate with M/1,000 HCl.

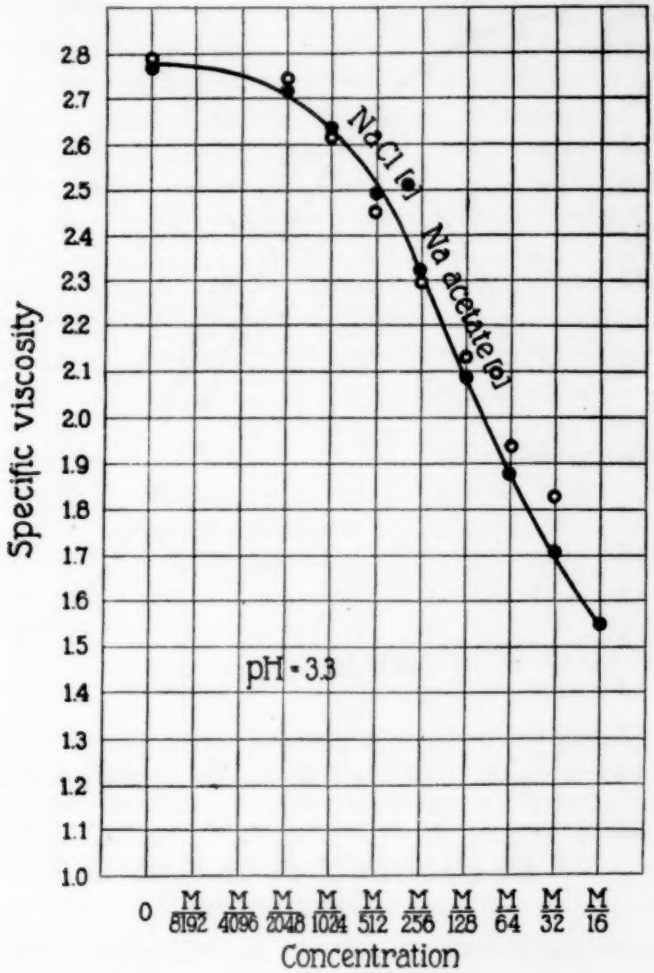

FIG. 9. When the pH is kept equal the depressing effect of equal concentrations of NaCl and Na acetate on the specific viscosity of a 1 per cent gelatin chloride or gelatin acetate solution of pH 3.3 is the same.

The 2 per cent gelatin chloride solution of pH 3.0 was diluted with 50 cc. of this mixture so that the resulting 1 per cent gelatin chloride solution of pH 3.0 contained various concentrations of Na acetate (or

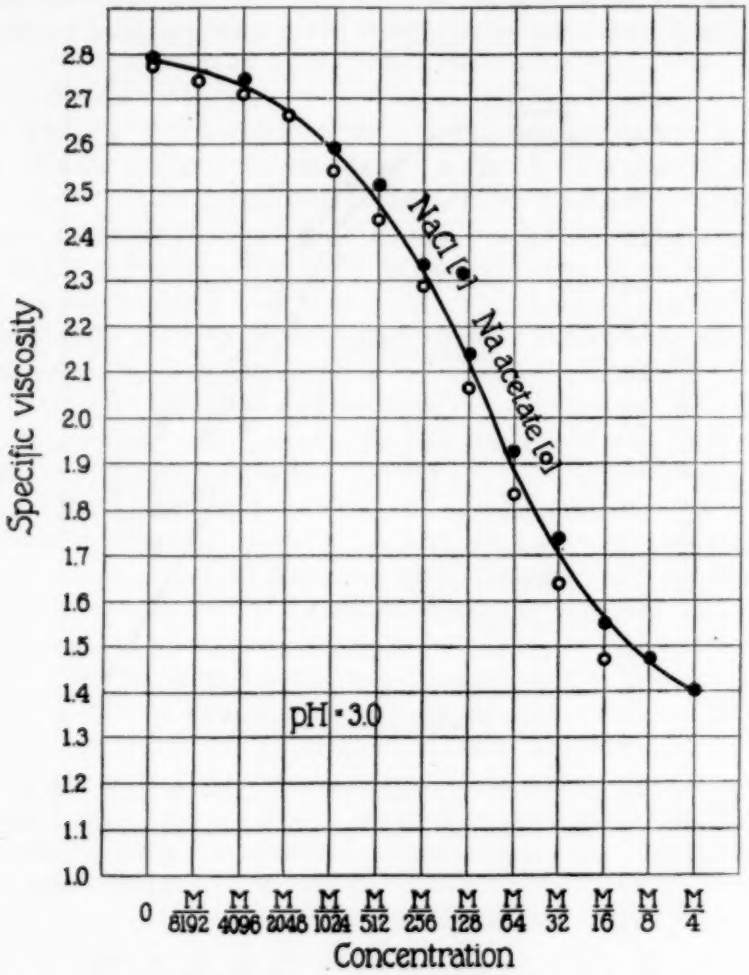

FIG. 10. See legend of Fig. 9, except that the pH of gelatin solution is 3.0.

more correctly of NaCl and Na acetate). The curve representing the depressing effect of this salt is given in Fig. 10, and is shown to be identical with the curve representing the depressing effect of the addition of NaCl to gelatin chloride of pH 3.0.

We can, therefore, state that sodium acetate has the same effect on the viscosity of gelatin chloride as the addition of any other salt with monovalent anion, and that the anomalous effect ascribed to the acetate anion in the colloidal literature is in reality due to the depression of the hydrogen ion concentration of the gelatin solution by the Na

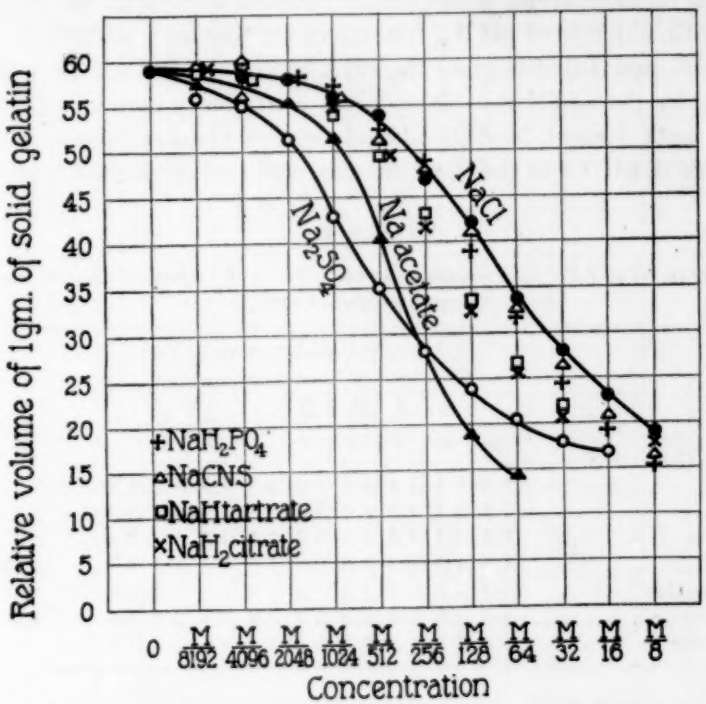

FIG. 11. Showing that the depressing effect of salts with monovalent anion on the swelling of gelatin chloride of pH 3.3 is similar to that on the specific viscosity. All salts with monovalent anion depress the swelling of gelatin chloride to the same extent, the seeming deviation from this rule being due to variation in the pH of the gelatin solution caused by buffer salts.

acetate which is a buffer salt. The failure to recognize the buffer character of salts, like the acetates, citrates, and tartrates, has led to the error of the Hofmeister ion series. In reality we find our valency rule confirmed whereby all salts with an anion of the same valency have the same relative depressing effect on the viscosity of a gelatin chloride solution if the pH of the solution is kept constant.

What has been demonstrated for the effect of these salts on the viscosity of gelatin solutions holds also for their effect on the swelling of gelatin. The same volumetric method for measuring the swelling effect was used which was described in the preceding paper. Fig. 11 gives the relative depressing effect of NaCl, NaH_2PO_4, NaCNS, monosodium tartrate, monosodium citrate, and Na acetate on the swelling of gelatin chloride of pH 3.3 (the curve for Na_2SO_4 is added for comparison), and Table II gives the variation of the pH of the gelatin caused by the addition of these salts. Our theory demands that all these salts (except Na_2SO_4) should depress the swelling of gelatin chloride of pH 3.3 to the same amount, and that deviations from this

TABLE II.

Changes in pH of 1 Per Cent Gelatin Chloride of pH = 3.3 upon Addition of Various Concentrations of Salts.

	Molecular concentrations of salts used.											
	0	M/8,192	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8
NaCl	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3
Na_2SO_4	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.35	3.4	3.5	3.6
NaH_2PO_4	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.4	3.5	3.6	3.7
NaCNS	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.35	3.4
NaH tartrate	3.3	3.3	3.3	3.3	3.3	3.4	3.5	3.5	3.6	3.7	3.7	3.7
NaH_2 citrate	3.3	3.3	3.3	3.3	3.3	3.4	3.5	3.6	3.8	3.85	3.9	3.9
Na acetate	3.3	3.3	3.3	3.4	3.45	3.5	3.8	4.3	4.8	5.2	5.4	5.5

rule must find their explanation in variations of pH caused by the addition of salt. Table II shows that the variations in pH are small for NaCl, NaCNS, and NaH_2PO_4 and hence the curves for the depressing effect of these three salts upon the swelling of gelatin are almost identical, as the valency rule demands. Monosodium citrate and tartrate have a greater depressing effect on the hydrogen ion concentration and Na acetate has a still greater depressing effect than these two salts. This explains the apparent deviation of the curves for these three salts from the valency rule.

Salts like disodium tartrate, disodium oxalate, and trisodium citrate offer an opportunity for an interesting test for our theory on account

of the difference between the electrolytic dissociation of weak dibasic or tribasic acids and the salts of the same acids. A weak dibasic

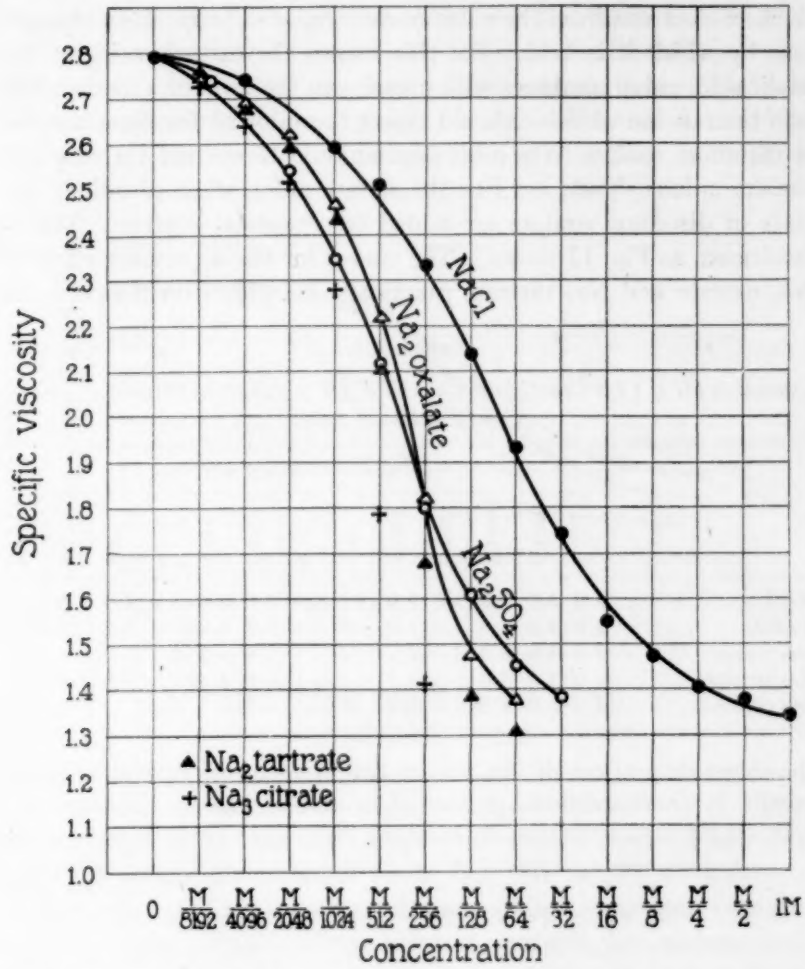

FIG. 12. Bivalent anions have an equally depressing effect as SO_4 on the specific viscosity of a 1 per cent gelatin chloride solution of pH 3.0.

acid like tartaric gives off one hydrogen ion easily but not both; sodium tartrate gives off both Na ions more readily. This is comprehensible on the idea that the oppositely charged ions in a molecule

are held together by electrostatic forces and that these forces are stronger in the case of a hydrogen ion which is free from electrons than in the case of a Na ion where the positive nucleus is separated by two shells of electrons from the valency electron of the oppositely charged ions by which it is held. For this reason the tartaric acid, or the oxalic acid, anion combines with proteins in the form of a monovalent acid tartrate ion while we should expect the anion of disodium tartrate or disodium oxalate to be a bivalent anion. Hence the tartrate and oxalate anions should act like the sulfate anion when disodium tartrate or disodium oxalate are added to a protein solution. This is confirmed, as Fig. 12 shows. The curves for the depressing effect of Na_2 oxalate and Na_2 tartrate practically coincide with the curve for

TABLE III.

Changes in pH of 1 Per Cent Gelatin Chloride of pH = 3.0 upon Addition of Various Concentrations of Salts.

	Molecular concentrations of salts used.												
	0	M/8,192	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8	M/4
NaCl	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Na_2SO_4	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.05	3.1	3.2	3.3	3.35
Na_2 oxalate	3.0	3.0	3.0	3.05	3.1	3.3	3.5	3.9	4.2	4.4			
Na_2 tartrate	3.0	3.0	3.0	3.1	3.2	3.3	3.7	4.0	4.35	4.7	4.8	5.0	
Na_3 citrate	3.0	3.0	3.05	3.2	3.3	3.8	4.4	5.2	5.7	6.1			

the depressing effect of Na_2SO_4 on the viscosity of gelatin solution, except in concentrations greater than M/256 where disodium tartrate and disodium oxalate cause also a depression of the hydrogen ion concentration (Table III) and where therefore the curves for these two salts drop more rapidly than the curve for Na_2SO_4. Hence disodium oxalate and disodium tartrate act like Na_2SO_4, while, as Fig. 8 shows, monosodium tartrate, monosodium citrate, and monosodium phosphate act like NaCl (if the necessary corrections for pH are made).

The osmotic pressure, viscosity, and swelling of Na gelatinate should be depressed by the cation of a salt and the more so the higher the valency of the cation. Fig. 13 shows that this is true for the swelling of Na gelatinate of pH about 9.3. The molecular concentration in

which the swelling is depressed by the same amount is about half as great for Na_2SO_4 as for NaCl (for molecular concentrations from M/256 to M/32), proving that the Na ion is responsible for the depression, while it is about eight times as high for NaCl as for $CaCl_2$. The pH of the gelatin was practically the same in all solutions.

All these data confirm our valency rule, whereby ions of the same valency and the same sign of charge have, in the same concentration, the same depressing effect on osmotic pressure, swelling, solubility in

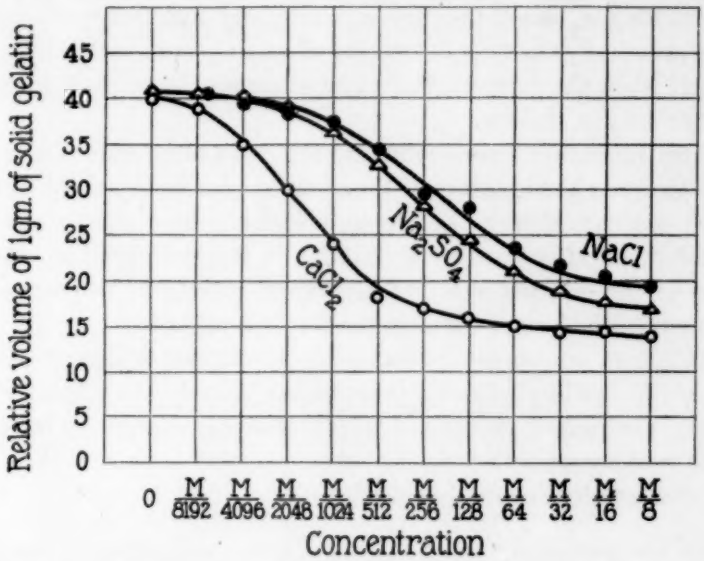

FIG. 13. The depressing effect of neutral salts on the swelling of Na gelatinate of pH about 9.3 is due to the cation of the salt, the depressing effect of NaCl being half as great as that of Na_2SO_4 of equal molecular concentration of Na_2SO_4 while that of $CaCl_2$ is considerably greater owing to the fact that Ca is bivalent.

alcohol, and viscosity of proteins; while the depressing effect increases rapidly with the valency. The Hofmeister ion series are due to the neglect of measuring the influence of the salts on the hydrogen ion concentration of the gelatin solutions. This neglect has given rise to the statement that salts, like sodium acetate, have the same depressing effect on the physical properties of proteins as the sulfates. A number of attempts to apply colloid chemistry to biology, pathology, and medicine are based on such errors.

SUMMARY.

1. Ions with the opposite sign of charge as that of a protein ion diminish the swelling, osmotic pressure, and viscosity of the protein. Ions with the same sign of charge as the protein ion (with the exception of H and OH ions) seem to have no effect on these properties as long as the concentrations of electrolytes used are not too high.

2. The relative depressing effect of different ions on the physical properties of proteins is a function only of the valency and sign of charge of the ion, ions of the same sign of charge and the same valency having practically the same depressing effect on gelatin solutions of the same pH while the depressing effect increases rapidly with an increase in the valency of the ion.

3. The Hofmeister series of ions are the result of an error due to the failure to notice the influence of the addition of a salt upon the hydrogen ion concentration of the protein solution. As a consequence of this failure, effects caused by a variation in the hydrogen ion concentration of the solution were erroneously attributed to differences in the nature of the ions of the salts used.

4. It is not safe to draw conclusions concerning specific effects of ions on the swelling, osmotic pressure, or viscosity of gelatin when the concentration of electrolytes in the solution exceeds M/16, since at that concentration the values of these properties are near the minimum characteristic of the isoelectric point.

Note.—The solutions of 1 per cent isoelectric gelatin were prepared by bringing 1 gm. of dry gelatin to the isoelectric point according to the method described in previous papers. It was found that in this process about 20 per cent of the gelatin was lost, so that the originally 1 per cent gelatin solution contained in reality only about 0.8 per cent isoelectric gelatin. This does not affect the contents of the paper or the conclusions since the gelatin concentration used was always the same.